Effect of water salinity on the hypophysis immunohistochemistry in mature *Liza ramada*

Original Article

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ABSTRACT

Introduction: The immunohistochemical description of the pituitary cell types were done in thin-lipped mullet, *Liza ramada*. Water quality plays a determined role in the activity of the pituitary gland of teleosts. Nevertheless, the immunohistochemistry of the hypophysis in mature *L. ramada* captivated in different waters was lacked.

Aim of the Work: We designed the present work to examine the environmental salinity effects on the immunohistochemistry of the hypophysis in mature *L. ramada*.

Material and Methods: Immunoreactivity of the hypophyseal hormone-secreting cells of mature *L. ramada*, captivated in fresh and saline waters, was investigated by the immunohistochemical technique using specific antibodies raised against hormones from piscine and mammalian origin.

Results: We investigated the immunoreactivities of the growth hormone family- cells (growth hormone; GH-, prolactin hormone; PRL- and somatolactin; SL- secreting cells) as well as the adrenocorticotropic hormone (ACTH) of mature *L. ramada* in both fresh water (FW) and saline water (SW). Mature brood fishes captivated in saline water exhibit a dramatic decrease in the area and size of GH-, PRL-, SL- and ACTH- immunoreactive (ir) cells in comparison to those of freshwater fishes. Also, the integrated optical density (IOD) of the GH family and the ACTH immunostaining inside the corresponding cells was declined significantly in saline water.

Conclusion: The observed changes in the immunoreactivities of PRL-, SL-, GH- and ACTH-ir cells, in response to increase in the environmental salinity, reinforces the potential role of such hormones in the osmoregulation during mullet aquaculture.

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Key Words: Immunohistochemistry, *liza ramada* (teleostei), pituitary gland, salinity, water quality.

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INTRODUCTION

The suitable water quality was essential for production of good quality of mature brood fish[1]. Water salinity plays an essential role in brood fish production; since it affects metabolism, fish performance[2]. With any change in the water salinity, the brood fish must regulate the osmosis with the lowest possible energy[3]. Improper water salinity can cause death and stress of fish, and long period of stress can impair the growth and immunity of fish as well as the maturation of the gonads[4;5].

The thin-lipped mullet, *L. ramada*, is euryhaline species that can matured in waters with different salinities ranged from fresh to full sea water[6;7]. However, *L. ramada* spawning requires full sea water[7; 8]. Similarly, the spotted scat, Scatophagus argus, brood fish require a salinity range of 15–25 ppt for gonad maturation[9] and their spawning need high range of salinity[10]. Also, gonadal maturation in Acanthopagrus butcheri was unchanged with water salinity changes[11].

In teleosts, the hypophysis arranges osmoregulation responses to alterations in water salinity by secreting GH/PRL/SL family hormones to manage the disturbance of salt and water balance[12;13;14]. Furthermore, the GH/PRL/SL family hormones also have been involved in the reproduction in several teleost species[15;16].

Also, ACTH has a regulatory role during sea water adaptation. The osmoregulatory role of the ACTH is possibly mediated by the interrenal secretion of cortisol[17;18]. In Oreochromis niloticus, the ACTH-ir cells, the melanin stimulating hormone (MSH) -ir cells and the SL-ir cells have a possible role during stress[19].

PRL is the adaptation hormone of bony fish to fresh water[20;21;22;23] and morphological investigations have showed inducement of PRL cells in several fishes[24] including Sparus auratus[25] and Mugil cephalus[26].

GH and cortisol, the conventional "SW-acclimate hormones" in fishes, forward the permanence in hyper-osmotic water sharing by provoking the acts of PRL[27;28]. GH appears to share in sea water (SW) acclimation[29] as manifested by morphological investigations explaining a trigger of GH producing-cells (GH cells) of SW-acclimated fish compared to freshwater fish (FW): Pungitius

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pungitius:[30]; Oncorhynchus kisutch[31]; Mugil cephalus[26]. However, the production of GH were increased in S. aurata acclimated to hypo-osmotic water[32].

Beside its role in osmoregulation[33], SL is implicated in stress response[34;35], acidosis[36], metabolism[12] and pigmentation in teleosts[37]. Furthermore, SL-immunoreactive cells displayed high activity during gonadal development and induction of spawning in captivated M. cephalus[38]. Furthermore, SL played a supposed role during adaptation to stress in *L. ramada*[35].

The aim of the present work was to assess the hypophysis activity of hatchery-reared thin-lipped mullet broodstock captivated in fresh and saline waters. The expected results will give essential knowledge for the production of mature fishes necessary for spawning in areas far away from the sea. This would help in mass production of seeds in this fish.

MATERIAL AND METHODS

Fish sampling and histological preparation

Specimens of *L. ramada*, selected for the present investigation, were obtained from saline water (35‰) ponds, at El-Matareyya Research Station and El-Serw freshwater (0.4‰) fish farm. 50 of mature L.ramada (both sexes; male and female), with standard length larger than 35 cm, were collected alive from each water type.

Before dissection, fishes were narcotized in clove oil at a dose of 40 mg/l. Standard total lengths and weights were measured for each fish. After dissection, the hypophysis connected to the brain was preserved, at 4◦C for 72 hr, in Bouin's fixative. The fixed samples were prepared histologically as before stated[38]. Sagittal serial sections of the pituitary glands were cutted at a thickness of 4 µm.

Immunohistochemical procedures

Antibodies: Human (h) ACTH antiserum was acquired from the National Institutes of Health (MD). Antibodies raised against Chum salmon hormones; GH, PRL and SL were obtained from Dr. H. Kawauchi (Kitasato University, Iwate, Japan).

Immunohistochemical technique

Immunohistochemical procedure for the pituitary sections was introduced with Avidin-biotin complex (ABC) Kit as previously illustrated[39]. In brief, hydrated sections were washed twice, for 10 min each, in phosphate-buffered saline (PBS; pH 7.4). All reactions of procedure were completed at the ambient temperature. Slides were washed in PBS after each step. The reaction of pituitary sections with the primary antibodies was keep for 12 hours. Experimentally, we acquired the used dilutions of the primary antibodies. Next, the pituitary slides were reacted for 1 h. with the secondary antibody, and for 45 min with AB-conjugated peroxidase. Thereafter, the immunoreaction was displayed with 3, 3`-diaminobenzidine tetrahydrochloride (DAB). At end, the immunohistochemical stained pituitary slides were completed as previously reported and mounting in DPX[39]. To confirm the specificity of the used antibodies, control sections of the pituitary were stained without the primary antibodies or replacement of the primary antibody with bovine serum.

Immunostaining semi-quantification

Semi-quantification for the immunostaining cells in the hypophysis was attained from five slides from each fish (ten samples from each water) cut at 4 µm. In brief, five sections from the midial of the hypophysis for each sample were applied for the measurement of cell size by light microscope (40X objective). The sizes of hormone-ir cells for each fish were cleared as the mean ± SD.

The immunostaining of the hypophysis sections were semi-quantified by the analysis software and Java Image processing (The software was downloaded from: http://rsb.info.nih.gov/ij/). The intensity of pixels and the area inside the threshold amounts performing immunoreaction were calculated, and the IOD (the output of the average of gray amount and the area) was enumerated. The IOD of fishes from different water types were compared and analyzed statistically.

Statistical Analysis

Differences between water types were examined by one-way ANOVA employing the water type as a variance factor. Tukey test was applied to distinguish significantly various values. The significantly differences were considered at $P<0.05$

RESULTS

The growth hormone family-immunoreactive (ir) cells (GH-, PRL- and SL- secreting cells) and the ACTH-ir cells of mature *L. ramada* changed with the salinity of water and displayed variations in their size, number, and immunoreactivity (Figures 1–18).

The ACTH- ir cells of mature L. ramada reared in different salinities

The ACTH-ir cells, which arrange as ribbon between the neurohypophysis (NH) and the PRL-ir cells, immunoreacted positively with human ACTH antibody (Figures 1,3). In response to salinity increasing in saline water (SW), the ACTH-ir cells had low number, size, and immunostaining in comparison to fresh water (FW) (Figures 2,4). Consequently, the IOD of the ACTH-ir cells for fishes reared in SW decreased by 18.22% than those of FW fishes ($P < 0.05$) (Table 1).

The MSH-ir cells of mature L. ramada reared in different salinities

The MSH-ir cells gave cross-reaction with anti-h ACTH (Figures 5,6). In saline water, the immunoreactivity of MSH-ir cells decreased, beside the decrease in both their number and size (Figure 6). The IOD of MSH immunostaining was reduced significantly by 30.36% for fish reared in SW than those of FW-reared fishes ($P < 0.05$, one-way ANOVA, Tukey test) (Table 1).

The PRL-ir cells of mature L. ramada reared in different salinities

The PRL-ir cells, which cover the main part of the pituitary rostral pars distalis (RPD), had specific immunoreaction with chum salmon PRL antibody (Figures 7-10). The production of PRL in SW fishes was decreased as reflected by the significant decrease (P<0.05) in their area and size (Figures 9,10). However, the IOD of the PRL immunoreactivity for fish reared in SW was slightly increased by 2.9% compared to that of FW (P<0.05, one-way ANOVA, Tukey test) (Table 1).

The GH-ir cells of mature L. ramada reared in different salinities

The GH-ir cells are situated, in the proximal pars distalis (PPD) of the hypophysis, as ribbons in association with neurohypophysis (Figures 11-14). They gave specific immunoreaction with chum salmon GH antibody (Figures 11-14). In SW, the activity of the GH-ir cells decreased in comparison to that of FW; as indicated by the significant decrease (P<0.05) in their size, area and immunoreactivity (Figures 13,14). Based on that, the GH-ir cells had significantly lower IOD (51.71±2.45) than that of FW fishes (79.93±3.80) (P<0.05, one-way ANOVA, Tukey test) (Table 1).

The SL-ir cells of mature L. ramada reared in different salinities

The SL-ir cells, which exhibit alterable shapes and sizes and detected in groups or singly in the PI, had specific immunoreaction with chum salmon SL antibody (Figures 15-18). The numbers, sizes and immunoreactivity of the SL-ir cells of fishes reared in SW were significantly decreased (P<0.05) in comparison to those of FW fishes (Figures 15-18). Accordingly, the SL-ir cells gave significantly lower IOD (34.13±1.45) compared with that of FW fishes (60.26±3.30) (P<0.05, one-way ANOVA, Tukey test) (Table 1).

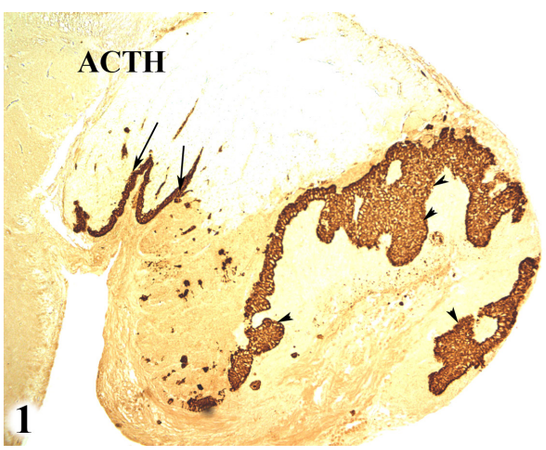

Fig. 1: Sagittal section in the hypophysis of mature L. ramada, reared in fresh water, immunostained with anti-h ACTH; showing the immunoreactive ACTH cells (arrows) and cross-reactive PbH+ cells in the PI (arrowheads). X40.

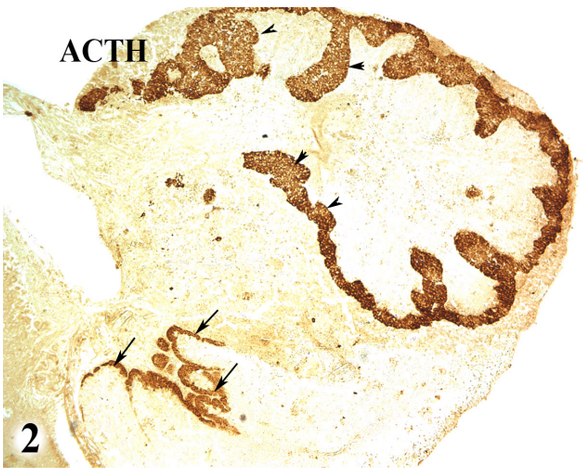

Fig. 2: Sagittal section of mature L. ramada hypophysis, reared in saline water, immunostained with anti-h ACTH; showing the immunoreactive ACTH cells (arrows) and cross-reactive PbH+ cells in the PI (arrowheads). X40.

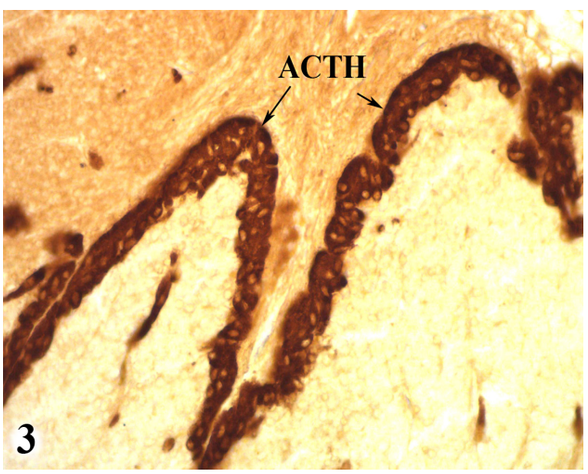

Fig. 3: A magnified portion of figure (1) showing strong immunoreaction of ACTH cells. X400.

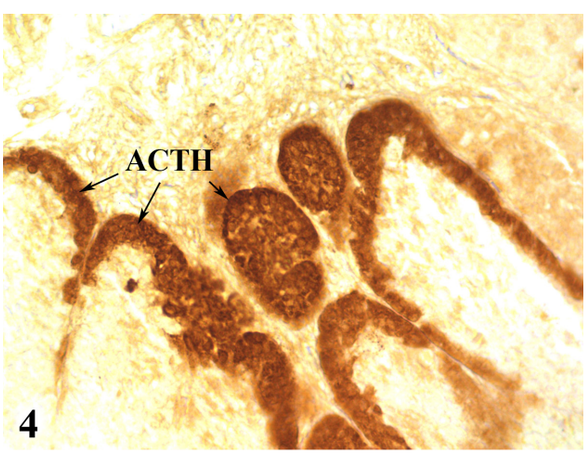

Fig. 4: A magnified portion of figure (2) showing strong immunoreaction of ACTH cells. X400.

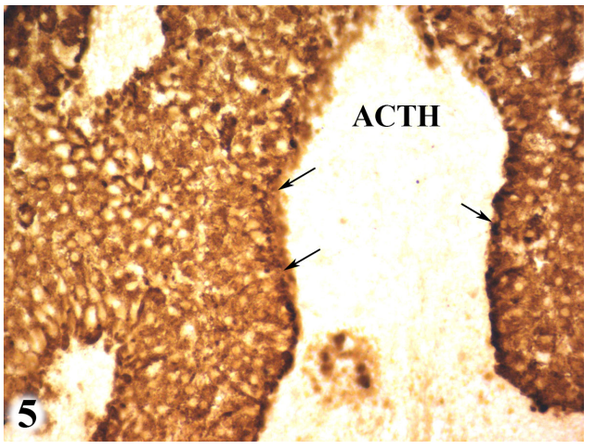

Fig. 5: A magnified portion of the section in figure (1) showing the PbH+ cells (arrows), in the PI of freshwater fish, displaying cross immunoreaction with anti-h ACTH antiserum. X400.

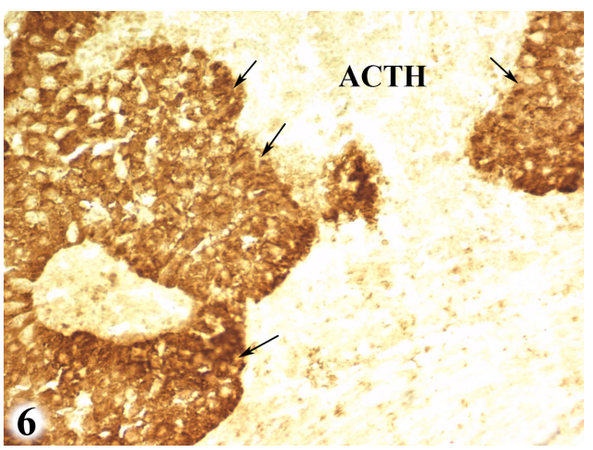

Fig. 6: A magnified portion of the section in figure (2) showing the PbH+ cells (arrows), in the PI of saline water fish, displaying cross immunoreaction with anti-h ACTH antiserum. X400.

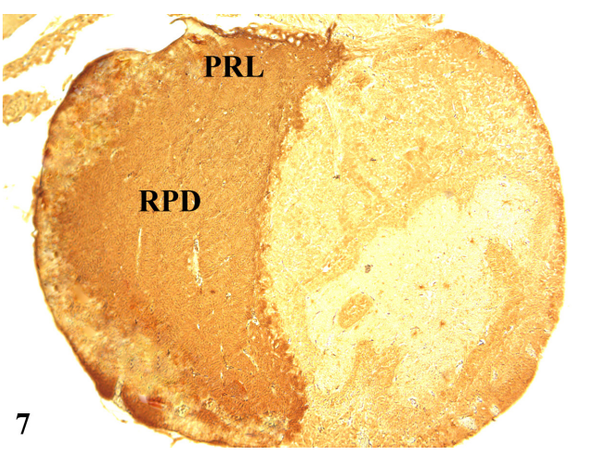

Fig. 7: Sagittal section in the hypophysis of L. ramada, reared in fresh water, immunoreacted with anti- salmon PRL antiserum. The PRL-ir cells are founed in the RPD. X40.

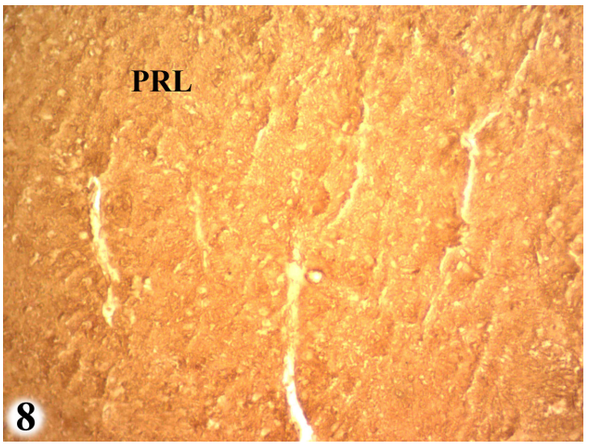

Fig. 8: A magnified portion of the section in figure (7) showing strong immunoreaction of PRL cells in the RPD of freshwater fish. X400.

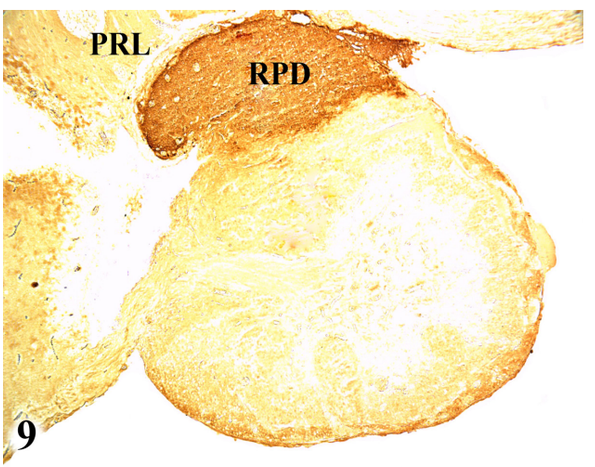

Fig. 9: Sagittal section of L. ramada hypophysis, reared in saline water, immunoreacted with anti- salmon PRL antiserum. PRL cells are found in the RPD. X40.

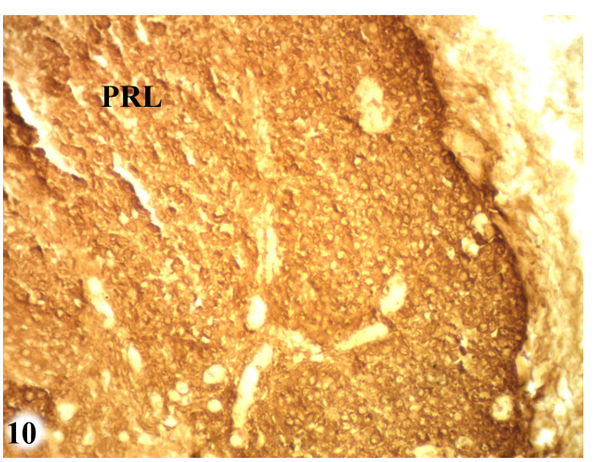

Fig. 10: A magnified portion of the section in figure (9) showing strong immunoreaction of PRL cells in the RPD of saline water fish. X400.

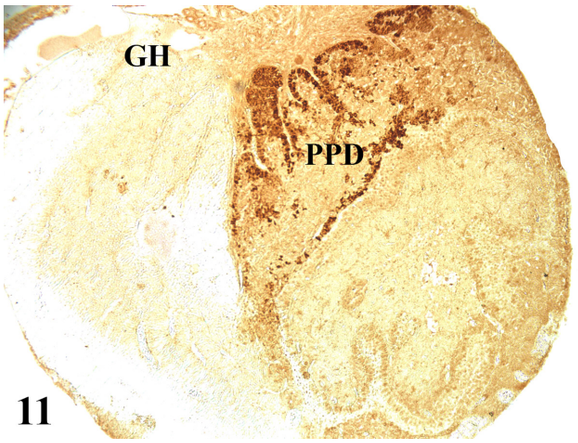

Fig. 11: Sagittal section of the pituitary gland of L. ramada, reared in fresh water, immunoreacted with salmon GH antiserum. The GH-ir cells are located in the PPD. X40.

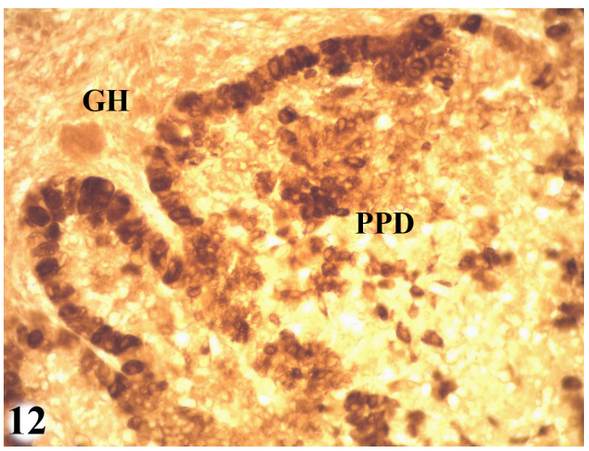

Fig. 12: A magnified portion of figure (13) showing the GH cells immunostained strongly with anti-chum salmon GH antiserum in PPD of freshwater fish. X400.

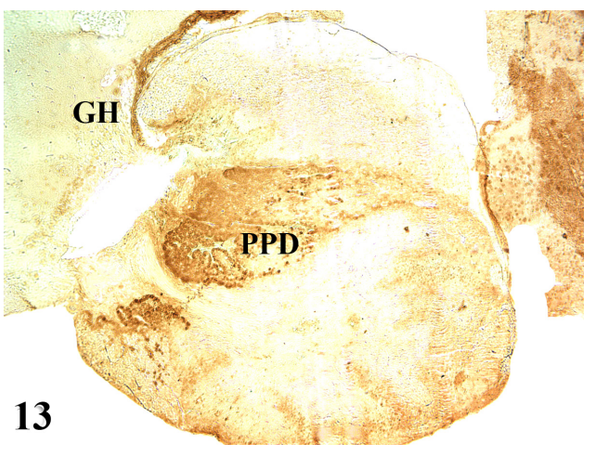

Fig. 13: Sagittal section of the pituitary gland of L. ramada, reared in saline water, immunoreacted with salmon GH antiserum. The GH-positive cells are located in the PPD. X40.

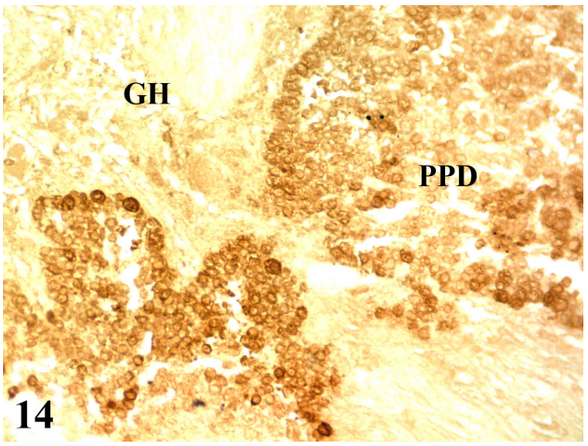

Fig. 14: A magnified portion of figure (13) showing the GH cells immunostained strongly with anti-chum salmon GH antiserum in PPD of saline water fish. X400.

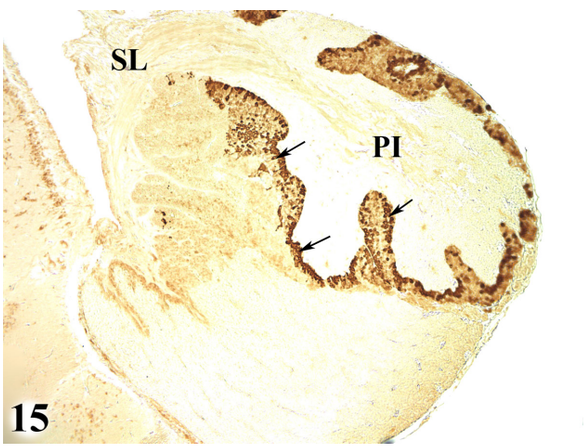

Fig. 15: Sagittal section of the pituitary gland of L. ramada, reared in fresh water, immunoreacted with salmon SL antiserum. The SL-ir cells (arrows) are located in the PI. X40.

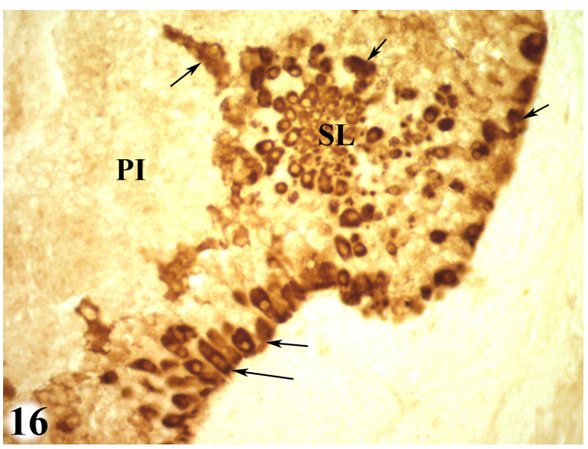

Fig. 16: A magnified portion of figure (13) showing the SL cells immunostained strongly with anti-chum salmon SL antiserum in PI of freshwater fish. X400.

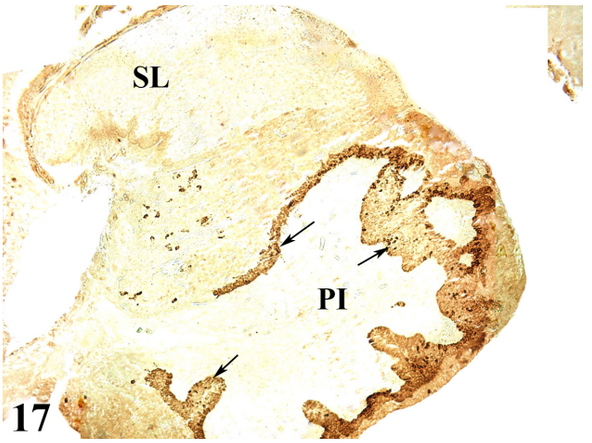

Fig. 17: Sagittal section of the pituitary gland of L. ramada, reared in saline water, immunoreacted with salmon SL antiserum. The SL-positive cells (arrows) are located in the PI. X40.

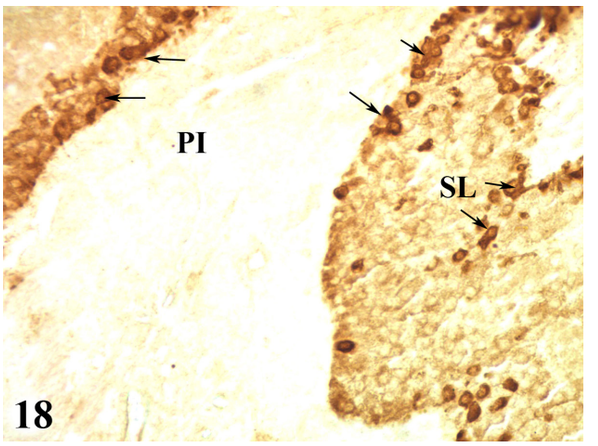

Fig. 18: A magnified portion of figure (13) showing the SL cells (arrows) immunostained strongly with anti-chum salmon SL antiserum in PI of saline water fish. X400.

Table 1: The immunoractivity of GH family-ir cells (GH-, PRL- and SL-ir cells) and the ACTH-ir cells; cell number, cell size (µm2), integrated optical density (IOD) and IOD% of SW (% from FW) of mature *L. ramada* reared at different waters; fresh water (FW) and saline water (SW)

Hypophyseal-ir cell type	Water type	Hypophyseal-ir cell immunoreactivity			
		Cell number	Cell size	IOD	IOD%
ACTH-ir cells	FW	915±32	75±3.55	73.80±4.30	100.00
	SW	1805±43 a	100±2.30 a	60.35±3.45 a	81.78
MSH-ir cells	FW	5125±255	110±4.55	84.45±5.25	100.00
	SW	3720±230 a	135±5.30 a	58.81±2.65 a	69.64
PRL-ir cells	FW	15050±1250	100±3.45	87.68±5.85	100.00
	SW	5120±300 a	70±2.70 a	90.22±6.35	102.90
GH-ir cells	FW	2705±250	100±3.75	79.93±3.80	100.00
	SW	3225±300 a	65±2.20 a	51.71±2.45 a	64.69
SL-ir cells	FW	1910±38	75±3.23	60.26±3.30	100.00
	SW	970±52 a	100±3.53 a	34.13±1.45 a	56.64

a: Significant differences when compared to FW (P<0.05).

DISCUSSION

The current immunohistochemical observations indicated that the ACTH-ir cells and GH family-immunoreactive (ir) cells of mature *L. ramada* responded to the changes of water salinity and gave different size, number, and immunoreactivity. The ACTH, produced in teleost hypophysis, has been implicated in osmoregulation control; since ACTH therapy in hypophysectomized teleost has been led to stimulate freshwater adaptation or to return plasma levels of sodium[18;19;40]. The activation of ACTH-ir cells in reaction to stress, as variations of salinity, was obtained[18;19;40]. The present results indicated that long-term reactions of the ACTH-ir cells to high salinity are apparently sufficient to create a rise in number and size of these cells in *L. ramada*. In addition, the exposure of *L. ramada* to high salinity is accompanied by a reduction in the ACTH-ir cells immunoreactivity suggesting secretion of ACTH. Similar finding was obtained in Pungitius pungitius[30]. However, the activities of the ACTH-ir cells are similar in both freshwater- and saltwater-reared Cynolebias whitei; so the role of the ACTH-ir cells in the osmoregulation is similar in both environments[41]. The role of ACTH in osmoregulation in fishes is probably mediated mainly by inter-renal secretion of cortisol[17;18].

In the present study, the exposure to saline water caused a dramatic decrease in the activity of PRL, GH and SL-ir cells. This indicated from the decrease in the area occupied with them and/or decrease in their sizes and the IOD. The immunostaining intensity of the PRL-ir cells shows that the content of these cells from PRL is slightly higher in saltwater-reared *L. ramada* than in the PRL-ir cells of the fishes from fresh water. Differences in the pituitary PRL content have been reported for C. whitei[41]; M. cephalus[26] and S. aurata[42] from different salinities. The results indicated that the production of the PRL from the PRL-ir cells, in saline water-reared *L. ramada* was reduced than that of FW as indicated by the lowering in their sizes and area. PRL forwards adaptation to FW by acting on conservation of ions by gills, kidneys and gastrointestinal tract[21;23]. In freshwater adapted fish, PRL possibly participate in the

permeability arrangement of gill and body surface[43]. In tilapia, PRL is in control of ionic equilibrium in low salinity waters[19;44;45;46]. Also, PRL sharing in osmoregulation was obtained during the acclimation to low salinity water in M. cephalus[47]; Pungitius pungitius[30] and S. aurata[48].

Otherwise, the GH promotes adaptation of fishes to sea water, partially, with the advancement of ionic extrusion in the gills[27;49]. The immunohistochemical observations showed that the production of GH was enhanced in *L. ramada*, inhabiting saline water, as indicated by the decrease in their sizes and immunoreactivity compared to that of FW. In contrast, the production of GH was increased in S. aurata and Pungitius pungitius adapted to hypo-osmotic environment[30;31]. Our results received a good support from previous study on tilapia; as GH treatment increase the survival rate in sea water and maintain mineral balance after hypophysectomy[29], which in turn increases the efficacy of tilapia to salt tolerance[19]. Also, GH has been illustrated to apply an anti-apoptotic action in teleost cells, in response to chemical stress, in the event that GH can work for protection[50].

In the present study, the immunoreactivity of SL-ir cells of *L. ramada* in saline water was lower than that in FW as reflected by their small area and weak immunostaining. Similarly, expression of SL in freshwater was significantly higher than in brackish water in both yellow and silver eels[14]. This proposes the role of SL in acclimatization to freshwater in eels. Although the information on physiological function of SL is limited, it has been reported that SL has been linked to several physiological functions including seawater acclimation[35], reproduction[35;38;39], stress[19;51], Ca-regulation[52] and acid–base balance[36;53]. The GH/PRL/SL family hormones also have been implicated in reproduction in several fishes[15;16;38;39;54].

The above mentioned attentions are of special link to water salinity, which induced changes in hypophyseal cells immunoreactivity, may be a participate factors in the reproduction of mullet populations; especially with regard to acclimation and coping with stress. Therefore, water salinity taken into consideration during mullet reproduction in different salinities.

CONFLICT OF INTERESTS

There are no conflicts of interest.

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الملخص العربى

تأثير ملوحة المياه على الهستوكيميائية المناعية للغدة النخامية لسمكة الطوبار

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المقدمة: تم وصف الهستوكيميائية المناعية للخلايا المفرزة فى الغدة النخامية لأسماك الطوبار. تلعب نوعية المياه دورا محددا فى نشاط الغدة النخامية للأسماك العظمية. مع ذلك؛ فإن الهستوكيميائية المناعية للغدة النخامية لأسماك الطوبار الناضجة المرباه فى الأسر فى مياه مختلفة لم يتم دراستها.

الهدف من البحث: يهدف هذا البحث إلى دراسة تأثير ملوحة المياه على الهستوكيميائية المناعية للغدة النخامية لأسماك الطوبار الناضجة.

المادة والطرق: تم التعرف على نشاط الخلايا المفرزة للهرمونات فى الغدة النخامية لأسماك الطوبار الناضجة باستخدام الطرق الهستوكيميائية المناعية والاستعانة بالأمصال المضادة لهرمونات الغدة النخامية للأسماك والثدييات.

النتائج: تم فحص نشاط خلايا عائلة هرمون النمو؛ هرمون النمو، هرمون البرولاكتين وهرمون السوماتولاكتين إضافة إلى الخلايا المفرزة لهرمون الأدرينوكورتيكوتروبين فى الغدة النخامية لأسماك الطوبار الناضجة فى كل من المياه العذبة والمياه المالحة. وجد إنخفاض ملحوظ حجم ومنطقة الخلايا المفرزة لعائلة هرمون النمو والخلايا المفرزة لهرمون الأدرينوكورتيكوتروبين فى الغدة النخامية لأسماك الطوبار الناضجة فى المياه المالحة مقارنة بتلك المرباه فى المياه العذبة. أيضا إنخفضت الكثافة الضوئية المتكاملة لصبغة الخلايا المفرزة لعائلة هرمون النمو والخلايا المفرزة لهرمون الأدرينوكورتيكوتروبين فى الغدة النخامية لأسماك الطوبار الناضجة فى المياه المالحة.

الخلاصة: تعزز التغيرات الناتجة فى نشاط الخلايا المفرزة لهرمون النمو، هرمون البرولاكتين وهرمون السوماتولاكتين إضافة إلى الخلايا المفرزة لهرمون الأدرينوكورتيكوتروبين، أثناء الاستجابة لارتفاع ملوحة المياه، الدور المحتمل لتلك الهرمونات فى تنظيم الأزموزية أثناء تربية أسماك الطوبار.