



Stress control during handling and confinement of the mature female *Liza ramada* by using adrenocortical inhibitor

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ABSTRACT

This study describes the effect of the adrenocortical inhibitor (metyrapone) on the activity of the brain-hypophyseal-interrenal system of *Liza ramada* mature females after handling and confinement stress. Adrenocorticotropin hormone (ACTH) and corticotrophin-releasing factor (CRF) are immunocytochemically localized in the pituitary and brain, respectively, and this suggests that CRF may mediate the ACTH release. During metyrapone administration, the brain's CRF-immunoreactive cells displayed an increase in their secretory and synthetic activity. During metyrapone administration to *L. ramada* mature females, the secretory activities of CRF-immunoreactive cells showed an increase after the first and second injections as reflected by degranulation and vacuolization. The secretory activity of injected females with 100 mg/ kg metyrapone was higher than that observed for the dose of 20 mg/ kg metyrapone. Also, ACTH-immunoreactive cells showed an increase in secretory activity during the first two injections as indicated by weak immunoreactivity. After the third injection of metyrapone, the synthetic activity of both CRF- and ACTH-immunoreactive cells was augmented as indicated by the increase in the immunoreactive staining intensity and granulation. Handling and confinement stress significantly increased the cortisol levels in both *L. ramada* control females and females injected with high doses of metyrapone, however low doses of metyrapone decreased cortisol levels. Therefore, when using the drug to examine the functional role of glucocorticoids, attention should be paid to these properties. It is concluded that metyrapone is suitable for stress control during the handling and confinement of *L. ramada* prespawning female.

INTRODUCTION

Inducing the maturation and ovulation of female mullet (*Liza ramada*; *M. cephaus*) with human chorionic gonadotropin (HCG) is successful, but pre-oviposition mortality was high and many fish did not oviposit as expected (Mousa, 1994; Mousa, 1999; Mousa *et al.*, 2013a). Knowing the potential causes of fish pre-oviposition mortality and the developing of effective technology for hatcheries depend on an understanding of the fish's physiology during the cycle of reproduction, acclimatization to seawater, and induction of spawning (Khalil *et al.*, 2012; Mousa *et al.*, 2013b; Shinde and Ganesh, 2022). During gonadal maturation and induction

of spawning, mature breeders of *L. ramada* respond to stress with elevated cortisol levels (Mousa and Mousa, 2006).

Pituitary ACTH takes a central role in the vertebrate stress response. When a fish meets stressful conditions, its brain-pituitary-interrenal axis is activated; this axis is the teleostean equivalent of the hypothalamo-pituitary-adrenal axis of mammals (Mousa and Mousa, 2006). Neurons in the different regions of the brain are activated to release corticotropin-releasing hormone (CRH). Consequently, the corticotrope cells secrete ACTH, the principal stimulator of cortisol secretion from the interrenal cells (Mousa and Mousa, 2006). In addition, the treatment of ACTH activated detached scales of *Dicentrarchus labrax* to generate and secrete cortisol (Samaras and Pavlidis, 2022). Moreover, in the presence of 11-beta hydroxylase inhibitor (metyrapone) which prevents synthesis of cortisol, the secretion of ACTH-derived cortisol was stopped from detached scales (Samaras and Pavlidis, 2022). Cortisol plays an adaptive role during stress in fish (McCormick, 1995; Wendelaar Bonga, 1997; Vijayan *et al.*, 1997; Mommsen *et al.*, 1999). Cortisol is responsible for the redistribution of energy flows, required to cope with the stressor (Wendelaar Bonga, 1997). CRH is considered to be the dominant stimulatory factor and key in ACTH release during acute stress responses (Rotllant *et al.*, 2000; Van Enckevort *et al.*, 2000; Mousa and Mousa, 2006). The receptors for CRH are expressed in fish gills, spleen and heart (Arai *et al.*, 2001; Pohl *et al.*, 2001) is in line with plasma CRH's peripheral effects. During times of stress, circulating CRH may control cardiac output. CRH receptors are clearly localized marginally in the atrium in *Oncorhynchus keta* and *Ameiurus nebulosus* (Arai *et al.*, 2001; Pohl *et al.*, 2001). Additionally, because CRH stimulates channel catfish (*Ictalurus punctatus*) activated peripheral leukocytes to secrete ACTH-ir in vitro; CRH may regulate circulating leukocytes during stress (Arnold and Rice, 2000). A decline in the activity of CRF-immunoreactive cells was observed during induction of spawning in *L. ramada* (Mousa and Mousa, 2006). Pre-oviposition mortality of ovulated females was accompanied with decrease in size and exhaustion of CRF-immunoreactive cells. Also, an increase in the secretory activity of ACTH-immunoreactive cells was recorded during ovulation of *L. ramada* (Mousa and Mousa, 2006). In addition, the exposure of *M. cephaus* during induction of spawning to stressors (handling, transportation and seawater acclimatization) significantly increased the mucous cell density in the gills during spawning. However, the mucous cell density in the mucosal layer of *M. cephaus* intestine was significantly decreased during spawning and at pre-oviposition mortality (Mousa *et al.*, 2013a). Furthermore, in the grey mullet, *M. cephalus*, the maturational steroid, 17 α , 20 β -dihydroxy-4-pregnen-3-one (17, 20-PG), is associated with final oocyte maturation and ovulation (Suzuki *et al.*, 1991). Barry *et al.* (1997) found that providing 17, 20-PG as a substrate could stimulate cortisol production by interrenal tissue from rainbow trout. With high levels of stress in ovulating women of *L. ramada*, high rate of cortisol production by interrenal tissue may increase the rate of 17, 20-PG sequestration which in turn affects the timing of ovulation, oviposition and spawning behavior of females (Mousa *et al.*, 2018).

Metyrapone, an inhibitor of 11-beta steroid hydroxylase which prevent the secretion of glucocorticoid during stress, is widely used to investigate the behavior and physiology of glucocorticoids (Samaras and Pavlidis, 2022). But metyrapone itself could work as stress. Thus, to test the use of the adrenocortical inhibitor (metyrapone) for control stress in *Liza ramada* mature females, the influences of different metyrapone doses on the activity of the brain-hypophyseal-interrenal system

were investigated in *Liza ramada* mature females after handling and confinement stress. CRF-like immunoreactivity in the brain and the adrenocorticotrophic hormone (ACTH)-like immunoreactivity in the pituitary were investigated immunocytochemically. In addition, the serum cortisol level was measured using an enzyme-linked immunoassay (ELISA).

MATERIALS AND METHODS

Study Site and Date:

The current study was conducted at El-Matareyya Research Station, from May 1, 2022, to January 30, 2023.

Fish:

The freshwater habitat of the culture ponds at El-Serw Fish Research Station was used to obtain the fish for this study. During *L. ramada* prespawning and spawning period (November to January), mature females with at least two years old were collected alive. These females exhibited weights ranging from 250 to 460 g and standard lengths ranging from 27 to 31.5 cm. *L. ramada* mature females were chosen with protruding genital papillae and a soft, swollen abdomen. During handling, the selected fish were put to narcotize with a solution containing 40 mg/l clove oil (Sigma). (Mousa, 2010).

Seawater Acclimation and Metyrapone Administration:

The experiments of metyrapone administration were conducted in *L. ramada*'s natural spawning season (December).

Ten fish per 1000-liter circular fiberglass tank were used to acclimate chosen breeders. Briefly, fish were moved to water with a salinity of 10 for 12 hours before it gradually rose to 32 for another 12 hours. Fish that had adapted were moved to 200-litre glass aquaria with continuously flowing seawater (32‰) and aeration (5 Females / aquarium) for injection with of the adrenocortical inhibitor (metyrapone, Sigma). The water temperature was between 19 and 21 °C. Metyrapone was dissolved in dimethylsulfoxide (DMSO). Three injections of metyrapone were administered to the fish by intraperitoneal injection, with doses of 20 or 100 mg/ kg of the body weight at interval of 24 h. Controls were injected only with DMSO. Zero control fish received no injections.

Tissue processing:

Immediately after the collection of the blood sample, the spinal cord was severed, just caudal to the head. The pituitary gland attached to the brain was removed and fixed at 4°C for 48 hours in Bouin's fluid. The fixed brain and pituitaries were then cleared and embedded in paraplast (M.P. : 56–58 °C) after being dehydrated through a graded ethanol solution. A series of 4 μm thick sections of the pituitary gland, connected to the brain, were taken at median sagittal plane.

Immunohistochemical procedures

Antibodies:

Rabbit antiserum directed against human ACTH was obtained from National Institute of Health. Rabbit anti-ovine CRF was kindly donated by Dr. Nigel Brooks (MRC Reproductive Biology Unit, Centre for Reproductive Biology, Edinburgh, Scotland).

Immunohistochemistry:

A vectastain ABC (avidin-biotin peroxidase complex) Kit (Vector Laboratories) was typically used for the immunocytochemical staining of the pituitary

gland and brain sections, as previously described (Mousa and Mousa, 1999). Briefly, sections were incubated overnight at 4 °C with the primary antibodies diluted 1:1000 for CRF and 1:500 for ACTH. The sections were then incubated for 1 hour with the biotinylated secondary antibody and for 45 minutes with avidin-biotin-conjugated peroxidase. The sections were then cleaned and stained for 3-5 minutes with 3,3-diaminobenzidine tetrahydrochloride (DAB). The sections were stained with Thionin as a counter stain after the enzyme reaction, dehydrated with successive applications of ethyl alcohol, cleaned in sulfur-free xylene, and installed in DPX.

In order to confirm the specificity of the immunoreactive procedures, adjacent sections were stained in accordance with the above-described protocol without the addition of the primary antiserum. Additionally, primary antiserum was substituted with normal bovine serum. In these areas, no cells or structures that were positive were discovered.

Samples of blood and analytical techniques:

Ten fish from each treatment group using a caudal severance, blood samples were taken. Fish were quickly removed from the aquarium to obtain blood samples, which took about 30 seconds for each fish. Blood was centrifuged after being placed in micro centrifuge tubes. Until necessary, the serum was separated and kept frozen at -20° C.

Serum cortisol was measured using an enzyme-linked immunoassay (ELISA) (Barry *et al.*, 1993 and 1995).

Statistical analysis:

Results were analyzed with the SPSS (Statistical Package for Social Sciences) statistical package. Paired-samples “t” test a method was used to compare means. $P \leq 0.05$ was the accepted statistical significance level.

RESULTS

The effect of metyrapone on the immunolocalization of CRF in *L. ramada* brain:

The activity of the CRF-immunoreactive neurons, in the medulla oblongata of brain, showed an increase during metyrapone administration to *L. ramada* mature females in compare with controls (Fig. 1a-h). The secretory activities of CRF-immunoreactive cells, of metyrapone-injected females, showed an increase after the first and second injections as reflected by degranulation (Figs 1b, c, f and g). The secretory activity of injected females with the dose of 100 mg/ kg metyrapone was higher than that observed for the dose of 20 mg/ kg metyrapone (Figs 1b, c, f and g). After the third injection of metyrapone, the synthetic activity of CRF-immunoreactive cells was augmented as indicated by increase in the immunoreactive staining intensity and granulation (Figs. 2d and h).

The effect of metyrapone on the immunostaining of ACTH in the pituitary gland of *L. ramada*:

During metyrapone administration to *L. ramada* mature females the secretory activities of ACTH-immunoreactive cells showed an increase after the first and second injections as reflected by degranulation (Figs 2b, c, f and g). The secretory activity of injected females with the dose of 100 mg/ kg metyrapone was higher than that observed for the dose of 20 mg/ kg metyrapone (Figs 2b, c, f and g). After the third injection, in both 20 mg/ kg and 100 mg/ kg metyrapone group, the synthetic activity of ACTH-immunoreactive cells was augmented as indicated by increase in the immunoreactivity, granulation, size and number (Figs. 2d and h). Also, an

increase in the synthetic activity of ACTH-immunoreactive cells was recorded in the control group received three injections of dimethylsulfoxide (Fig. 2e).

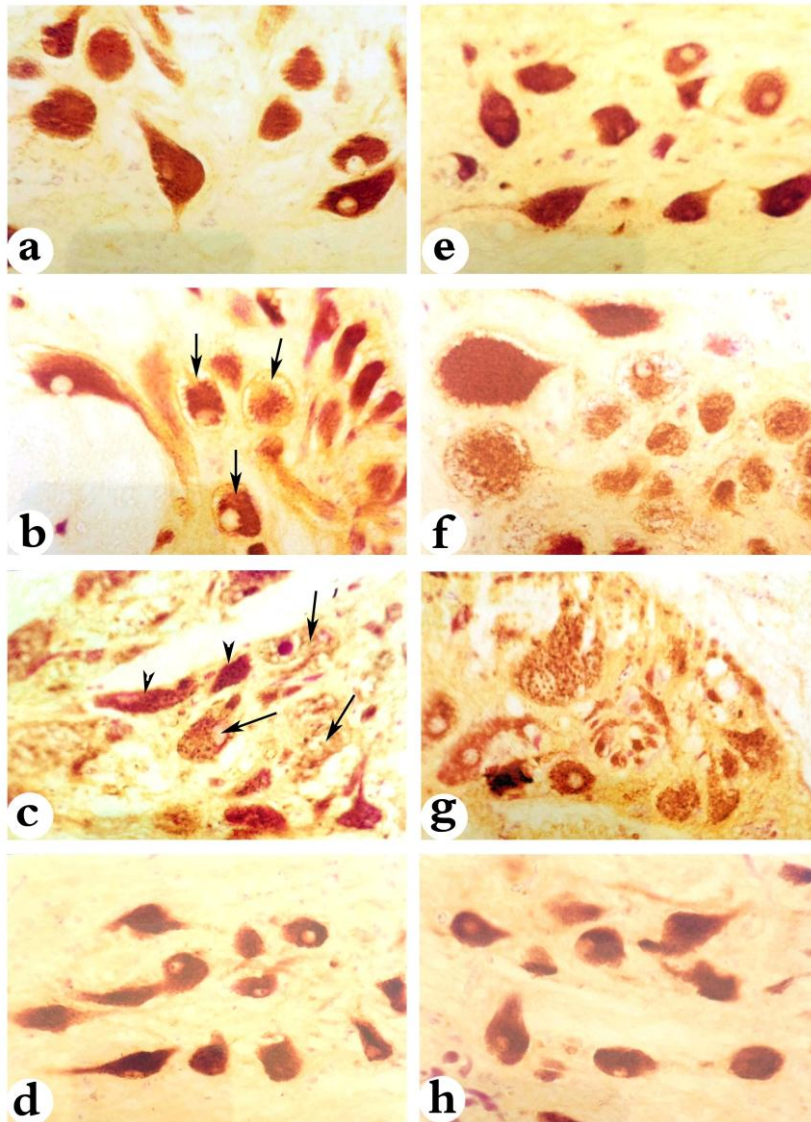


Fig. (1): Immunolocalization of CRF in the medulla oblongata (MO) of *L. ramada* mature females, from controls and injected groups. X400. (a) CRF-ir cells of control female, obtained at the start of the experiment (zero control), having different sizes with strong immunoreactivity. (b) CRF-ir cells of injected female, after one injection of 20 mg/ kg (24 h after the injection), exhibiting strong immunoreactivity and having secretory vacuoles (arrows). (c) CRF-ir cells of injected female, after two injections of 20 mg/ kg (48 h after the injection). Note the presence of both granulated (arrowheads) and degranulated cells (arrows). (d) CRF-ir cells of injected female, after three injection of 20 mg/ kg (72 h after the injection), are hypertrophied with strong immunoreactivity. (e) CRF-ir cells of control female, obtained at 72 h, are hypertrophied with strong immunoreactivity. (f) CRF-ir cells of injected female, after one injection of 100 mg/ kg (24 h after the injection). Note the increase of secretory activity as reflected by degranulation and decrease of immunoreactivity. (g) CRF-ir cells of injected female, after two injections of 100 mg/ kg (48 h after the injection), exhibiting different sizes and different degree of granulation. (h) CRF-ir cells of injected female, after three injection of 100 mg/ kg (72 h after the injection), having different sizes and strong immunoreactivity.

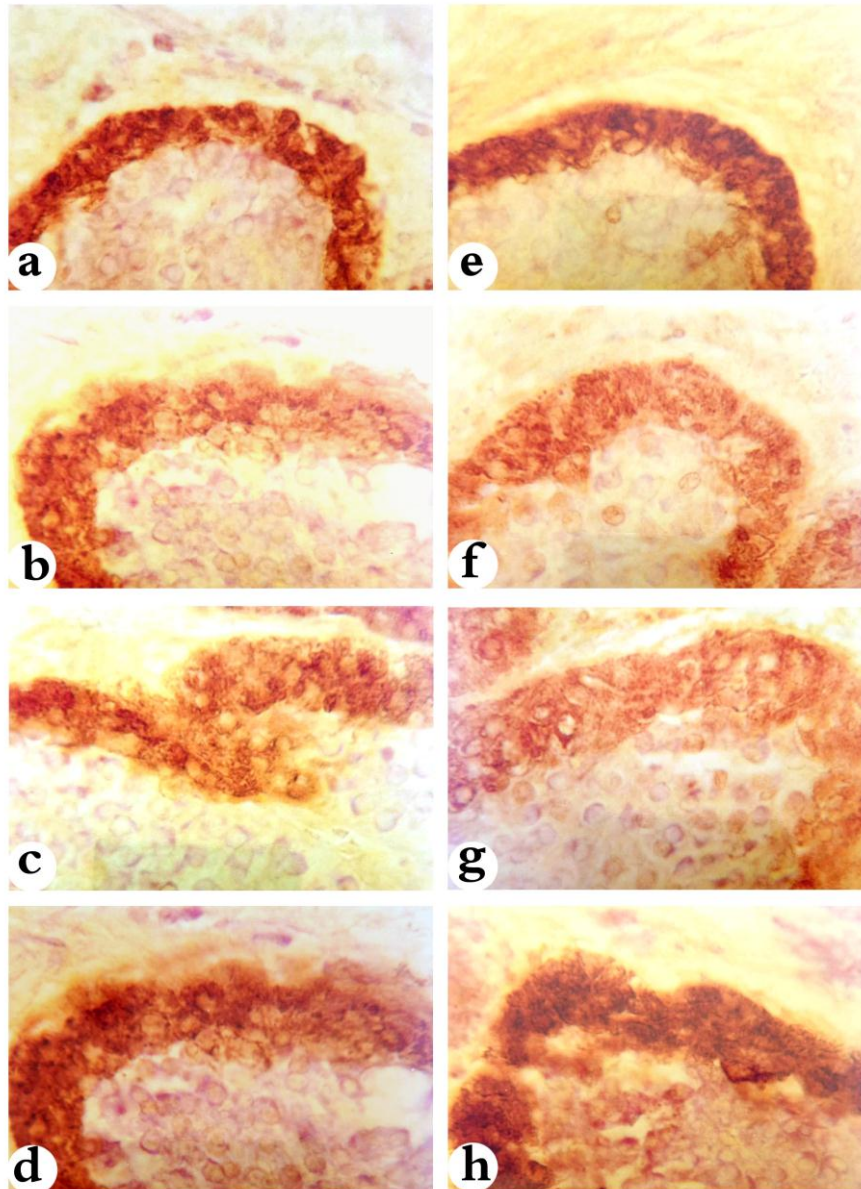


Fig. (2): Immunolocalization of ACTH in the pituitary of *L. ramada* mature females, from controls and injected groups. X400. (a) ACTH-ir cells of control female, obtained at the beginning of the experiment (zero control), having moderate immunoreactivity. (b) ACTH-ir cells of injected female, after one injection of 20 mg/ kg (24 h after the injection). (c) ACTH-ir cells of injected female, after two injections of 20 mg/ kg (48 h after the injection). Note the increase of secretory activity in (b) and (c) as reflected by degranulation and decrease of immunoreactivity. (d) ACTH-ir cells of injected female, after three injection of 20 mg/ kg (72 h after the injection), are hypertrophied with strong immunoreactivity. (e) ACTH-ir cells of control female, obtained at 72 h, exhibiting strong immunoreactivity. (f) ACTH-ir cells of injected female, after one injection of 100 mg/ kg (24 h after the injection). (g) ACTH-ir cells of injected female, after two injections of 100 mg/ kg (48 h after the injection). Note the increase of secretory activity in (f) and (g) as reflected by degranulation and decrease of immunoreactivity. (h) ACTH-ir cells of injected female, after three injection of 100 mg/ kg (72 h after the injection), having different sizes and strong immunoreactivity. X1000.

The effect of metyrapone on serum cortisol concentrations of *L. ramada*:

The mean serum cortisol concentrations in zero time controls and *L. ramada* injected with dimethylsulfoxide and metyrapone in dimethylsulfoxide are presenting in table (1) and figure (3). Handling and confinement stress significantly increased the cortisol levels in both *L. ramada* control females and females injected with the high dose of metyrapone, however low dose of metyrapone decreased cortisol levels. There was a progressive increase in mean serum cortisol concentration to 650 ng/ ml in the control (dimethylsulfoxide) group for three days, and to 710 ng/ ml in the 100 mg/ kg metyrapone group. However, in the group receiving three injections of 20 mg/ kg, the mean level of cortisol in serum was significantly decreased to 225.5 ng/ ml. The cortisol level in the group receiving three injections of 20 mg/ kg was significantly lower than the mean serum cortisol concentration in the control group and the 100 mg/ kg group ($P \leq 0.0005$).

Table (1): Serum cortisol concentrations (ng/ml) of both control and metyrapone-injected *Liza ramada* mature females sampling at 0 h (before injection), 24 h, 48 h and 72 h after injection.

Sampling Time	Control	20 mg / kg body weight	100 mg / kg body weight
0 h (bef. injection)	490.0±9.13	-	-
24 h	520.5±14.42	550.5±14.80*	600.0±31.27 ^a
48 h	550.5±15.54	500.5±12.79*	650.0±30.18*
72 h	650.0±18.41	225.5±29.39*	710.0±31.97*

Each value represents the mean±SD of 10 fishes.

Asterisks denote mean values significantly ($P \leq 0.0005$) different from that of controls at the same time.

a: denote mean values significantly ($P \leq 0.001$) different from that of controls at the same time.

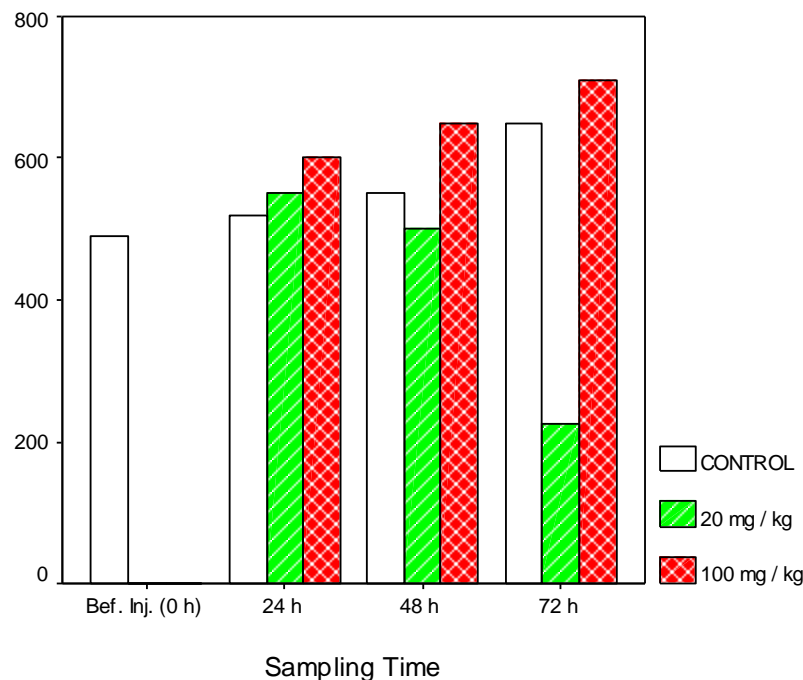


Fig. (3): Serum cortisol concentration of both control and metyrapone-injected *Liza ramada* mature females sampling at 0 h (before injection), 24 h, 48 h and 72 h after injection.

DISCUSSION

In the present work, the effects of various metyrapone dosages on the activity of the brain-hypophyseal-interrenal system were investigated in *Liza ramada* mature females after handling and confinement stress. Studies on the physiological and behavioral functions of glucocorticoids frequently use the 11-beta steroid hydroxylase inhibitor metyrapone, which inhibits stress-induced glucocorticoid release. Metyrapone blocks the enzyme 11 β -hydroxylase essential for cortisol synthesis in adrenal steroidogenesis pathway (**Ganesh et al., 2023**). Contradictory evidence, however, raises the possibility that metyrapone might function as a pharmacological stressor. The immunocytochemical localization of CRF in the brain indicated that the synthetic and secretory activity of the CRF-immunoreactive cells, showed an increase during metyrapone administration. During metyrapone administration to *L. ramada* mature females the secretory activities of CRF-immunoreactive cells showed an increase after the first and second injections as reflected by degranulation. The secretory activity of injected females with the dose of 100 mg/ kg metyrapone was higher than that observed for the dose of 20 mg/ kg metyrapone. Also, ACTH-immunoreactive cells showed an increase in the secretory activity during the first two injections as indicated by weak immunoreactivity. Similar observation obtained in the eel (**Oliveriau and Oliveriau, 1990**). The release of ACTH from the RPD is classically considered to be under positive control of CRF. Several studies have shown that CRF is a stimulator of ACTH release in fish (**Fryer et al., 1984; Oliveriau and Oliveriau, 1990; Baker et al., 1996; Rotllant et al., 2000; Van Enkevort et al., 2000**). CRF and ACTH's relative immunocytochemical locations in the brain and pituitary gland, suggests that CRF may mediate discharge of ACTH in *L. ramada*. After the third injection of metyrapone, the synthetic activity of both CRF-and ACTH-immunoreactive cells was augmented as indicated by increase in the immunoreactive staining intensity and granulation.

Handling and confinement stress significantly increased the cortisol levels in both *L. ramada* control females and females injected with the high dose of metyrapone, however low dose of metyrapone decreased cortisol levels. Cortisol is the main indicator to acute stress in fish (**Samaras and Pavlidis, 2022**). Elevated plasma cortisol levels increased mortality in female salmon *Oncorhynchus nerka* (**Eliason et al., 2020**). Decreased cortisol concentrations in females of *L. ramada* at low dosage level of metyrapone suggested that the drug of metyrapone was exerting a marked inhibitory effect on 11 β -hydroxylation. However, increased concentrations at high dosage level of metyrapone indicated that metyrapone was also stressful. The relatively high concentrations of cortisol observed in the control fish may have been a result of a stressful effect of dimethylsulfoxide, or of the daily injection procedure itself. Similar observation obtained in *Salmo gairdneri* (**Fagerlund et al., 1968**). In metyrapone groups, three injections of 100 mg / kg resulted in cortisol concentrations higher than those observed in dimethylsulfoxide or control groups, suggesting that the major effect of metyrapone at this dosage may have been that of a stressor, rather than an 11 β -hydroxylase inhibitor. However, three injections of 20 mg/ kg, resulted in mean cortisol concentrations lower than those of the control groups, showing that 11 β -hydroxylase inhibition was occurring. That metyrapone may be a stressor in animals has been indicated by **Rotllant et al. (2002)** who found that the drug at a dose of 200 mg/ kg evoked an increase in blood ACTH levels in male rats. In this respect, Rotllant and Armario (2005) observed similar stressful effect for metyrapone injected at the same dose. Thus, attention should be paid to these properties when using the drug to study the functional role of glucocorticoids.

Corticotropin-releasing hormone (CRH) is a regulator for the stress response (**Lu et al., 2004; Bernier, 2006**). The secretion of ACTH from the pituitary gland is stimulated by CRH, which stimulates the secretion of corticosteroids by interrenal tissue in fish (**Wendelaar Bonga, 1997; Charmandari et al., 2005**). During times of stress, circulating CRH may control cardiac output. In the atrium of *Ameiurus nebulosus* and *Oncorhynchus keta* (**Arai et al., 2001; Pohl et al., 2001**), CRH receptors are most widely expressed in the periphery. In addition, as CRH stimulates *Ictalurus punctatus* activated peripheral leukocytes

to secrete ACTH-ir in vitro, CRH may regulate circulating leukocytes during stress. (Arnold and Rice, 2000). The expression of CRH receptors in fish heart, gills, and spleen (Arai *et al.*, 2001; Pohl *et al.*, 2001) is consistent with the peripheral actions of plasma CRH. Also, corticotropin-releasing factor represents a role in the appetite regulation (Bernier, 2006). Thus, the deficiency of CRF in the circulation of ovulated females of *L. ramada* may be considered one of the possible reasons to the death of the fish before oviposition. The induction of CRF and ACTH synthesis and the inhibition of cortisol synthesis in the prespawning female, injected with low dose of metyrapone, may be necessary to ensure that a surge in both CRF and ACTH occurs during final maturation and spawning of *L. ramada*. Also, CRH has anti-steroidogenic effect in cultured *Danio rerio* follicular cells (Zhou *et al.*, 2021). Cortisol is also believed to prevent tissue damage by reducing the stress-induced inflammatory/immune response (Bamberger *et al.*, 1996). The hypersecretion of glycoproteins in response to stress early during handling and acclimation led to decreasing glycoprotein content in mucous cells in *M. cephalus*. This decreased the mucus production and reduced its protective role against desiccation of gill and intestine tissue when the ion concentration in water changed during induced spawning and may be considered one of the possible reasons for mortality (Mousa *et al.*, 2013a). Additionally, persistently high cortisol levels appear to be the root of the immunosuppression that makes fish sensitive to infections (Schreck *et al.*, 2001). Furthermore, in the grey mullet, *M. cephalus*, the maturational steroid, 17 α , 20 β -dihydroxy-4-pregnen-3-one (17, 20-PG), is associated with final oocyte maturation and ovulation (Suzuki *et al.*, 1991). Barry *et al.* (1997) found that cortisol production by interrenal tissue from rainbow trout could be stimulated by providing 17 α , 20 β -PG as a substrate. In *L. ramada*'s ovulated females under extreme stress, the high rate of cortisol production by interrenal tissue may increase the rate of 17 α , 20 β -PG sequestration which in turn affects the timing of ovulation, oviposition and spawning behavior of females. The negative stress impacts on fish reproduction have been recorded (Rousseau *et al.*, 2020). Similarly, cortisol has inhibitory effect on the gonadotropin-stimulated steroidogenic capacity in *Perca fluviatilis* (Mandiki *et al.*, 2017). In this respect, high cortisol levels reduced longevity together with a reduction in the eggs number released in *Oncorhynchus gorbuscha* (McConnachie *et al.*, 2012). Moreover, β -endorphin (derivative from proopiomelanocortin) has a role in stress response along inhibition of luteinizing hormone (LH) secreting cells–gonad axis in *Oreochromis mossambicus* (Ganesh and Chabbi, 2013; Chabbi and Ganesh, 2014; 2016; Shinde and Ganesh, 2022).

It is concluded that Metyrapone is suitable for induction of CRF and ACTH synthesis and the inhibition of cortisol synthesis in the prespawning female, when injected with low dose at 20 mg/ kg. This is necessary to ensure that a surge in both CRF and ACTH, which may be useful during final maturation and spawning of *L. ramada*.

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ARABIC SUMMARY

التحكم فى الإجهاد أثناء التداول والأسر للأمهات الناضجة لسمكة الطوبار
باستخدام مثبط إفراز هرمون الأدرينوكورتيكوتروبين

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فى هذا البحث تم دراسة تأثير الحقن بمادة المثيرابون على نشاط الجهاز المفرز للهرمونات المواجهة للإجهاد فى الأمهات الناضجة لسمكة الطوبار أثناء التداول والأسر.

يؤدى التميز لكل من العامل المحرر لهرمون الكورتيكوتروبين فى المخ وهرمون الأدرينوكورتيكوتروبين فى الغدة النخامية إلى الافتراض بأن العامل المحرر لهرمون الكورتيكوتروبين ربما يساعد على إفراز هرمون الأدرينوكورتيكوتروبين. أوضحت النتائج أن النشاط التخليقى والإفرازى للخلايا المفرزة للعامل المحرر لهرمون الكورتيكوتروبين يزداد أثناء الحقن بمادة المثيرابون. وتجدر الإشارة إلى أن الطاقة الإفرازية للخلايا المفرزة للعامل المحرر لهرمون الكورتيكوتروبين تزداد بعد الجرعة الأولى والثانية من الحقن بمادة المثيرابون كما اتضح من قلة التحبب وظهور فجوات إفرازية. كانت الطاقة الإفرازية للأمهات المحقونة بجرعة 100 مجم / كجم مثيرابون أعلى من تلك فى الأمهات المحقونة بجرعة 20 مجم / كجم مثيرابون. كما أوضحت النتائج السيتوكيميائية المناعية أن الطاقة الإفرازية للخلايا المفرزة لهرمون الأدرينوكورتيكوتروبين تزداد بعد الجرعة الأولى والثانية من الحقن بمادة المثيرابون كما اتضح من التفاعل المناعى الضعيف. أيضا أوضحت النتائج السيتوكيميائية المناعية أن الطاقة التخليقية لكل من الخلايا المفرزة للعامل المحرر لهرمون الكورتيكوتروبين والخلايا المفرزة لهرمون الأدرينوكورتيكوتروبين إزدادت بعد الجرعة الثالثة كما اتضح من كثرة التحبب والتفاعل المناعى القوى.

أدى الإجهاد الناتج عن عملية التداول والأسر للأمهات إلى ارتفاع معدل الدم من هرمون الكورتيزول فى كل من أمهات المجموعة الضابطة والمحقونة بالجرعة المرتفعة من المثيرابون، ولكن كان تركيز هرمون الكورتيزول منخفضا فى الأمهات المحقونة بالجرعة المنخفضة من المثيرابون. لذلك يجب توخى الحذر أثناء استخدام تلك المادة لدراسة الدور الوظيفى للهرمونات الخاصة بمواجهة الإجهاد.

يمكن استنتاج أن الحقن بمادة المثيرابون يكون مناسباً للتحكم فى الإجهاد أثناء أسر وتداول الأمهات الناضجة لأسماك الطوبار.