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Physiological State as a Time-Determining Factor for Obtaining and Handling Mature *Liza ramada* During the Spawning Season

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ABSTRACT

The physiological state of mature brood fish is a determining factor for achieving and stimulating a successful spawning process in captivity. In this 2- year investigation, the physiology of mature *Liza ramada* females was examined throughout the day at various times (24 hours) to ascertain the most suitable time for obtaining mature females raised in both saline water and fresh water in addition to handling them during the spawning season. In addition, blood concentrations of hormones involved in growth, acclimatization, and response to stress, as well as the levels of glucose, total protein, albumin, and the albumin: globulin ratio of *L. ramada* mature females were all investigated with respect to the factor of time during a period of 24hours. The highest levels of growth hormone, thyroxine, triiodothyronine, prolactin, cortisol, total protein and albumin were recorded at 12 midday and 6 in the morning for mature *L. ramada* females raised in both saline water and fresh water, respectively. These recorded levels were suitable for attaining a successful spawning and concurrently stimulating the process for *L. ramada* raised in captivity. Average levels of glucose were recorded for these fish at the aforementioned times. Low levels of the mentioned hormones, total protein, and albumin were observed at 6PM in fish raised in both saline and fresh waters. These hormones play a significant role in growth, acclimatization, stress response during handling, and stimulating spawning of *L. ramada*. In conclusion, it was proved that the optimal times for obtaining and handling mature *L. ramada* females during spawning induction are at 12PM for fish raised in saline water and 6AM for fish raised in fresh water.

INTRODUCTION

The biological rhythm known as the circadian pattern, or cycles of day and night, helps regulate a number of physiological functions. Pituitary and pineal gland hormone secretion are directly correlated with photoperiod. Cortisol and thyroid hormone levels fluctuate throughout the day and season, preventing physiological parameters like body temperature from deviating from normal. These two hormones are crucial for controlling the body's homeostasis, osmoregulation, metabolism, and reproduction **(Deal & Volkoff,**

2020; McCormick *et al*., 2020; Ranjbar & Mohammad Nejad, 2020; Zwahlen *et al*., 2024).

The cortex of the adrenal gland is stimulated by the anterior pituitary gland's adrenocorticotropin (ACTH), which releases cortisol, a glucocorticoid **(Angelousi *et al*., 2020; Shida *et al*., 2020)**. Cortisol is primarily engaged in the metabolic process of carbohydrates, fats, and proteins **(Wang *et al*., 2022)**. Since its levels rise during stressful situations, it could function as a potential gauge of an animal's overall wellbeing. Its role is to uphold the sympathoadrenal system, which manages the body's homeostasis **(Eckert *et al*., 1988)**. Glucocorticoids stimulate gluconeogenesis, which raises blood glucose levels by synthesizing glucose molecules primarily from the liver's conversion of fat and protein **(Han *et al*., 2022)**.

Growth hormone (GH) exhibits pluripotency, exhibiting a broad spectrum of effects beyond its well-known growth stimulatory properties in fish **(Björnsson, 1997)**. In addition to controlling animal growth, growth hormone (GH) has an impact on energy mobilization, development, nutritional requirements, and social behavior in fish **(Triantaphyllopoulos *et al*., 2020; Velez & Unniappan, 2021)**. Numerous studies have demonstrated that GH influences a range of behavioral traits with ecological implications, such as aggression, foraging behavior, appetite, and predator avoidance **(Yousefian & Shirzad, 2011; Canosa & Bertucci, 2023)**.

It is commonly known that the thyroid hormone regulates fish growth, differentiation, osmoregulatory effects, and regulation **(Peter *et al*., 2000; Deal & Volkoff, 2020; Seale *et al*., 2021)**. Thyroxine (T4) and triiodothyronine (T3) are the two thyroid hormones whose secretion is influenced by the activity levels as well. By speeding up the oxidation of glucose and raising the quantity of metabolic heat generated, T4 and T3 are primarily responsible for boosting metabolism **(Zwahlen *et al*., 2024)**. Thyroid hormones additionally support the growth of bones and tissues, as well as complete neural development **(Zwahlen *et al*., 2024)**. Depending on the animal species, thyroid hormone secretory patterns have been demonstrated to exhibit both seasonal and circadian rhythms **(Deal & Volkoff, 2020; Özeren *et al*., 2020; López-Olmeda *et al*., 2021)**.

Fish ionosomotic balance is controlled by several important hormones, including thyroxine (T4), triiodothyronine (T3), prolactin (PRL), cortisol and growth hormone (GH) **(Sakamato & McCormick, 2006; Yeşilbaş & Oğuz, 2022)**.

An essential phase in the advancement of mullet cultivation is the application of acute hormone injections as a technique for induced ovulation **(Mousa, 2010)**. Female mullet ovulation can occur with success by injecting chorionic gonadotropin, but mortality prior oviposition is elevated and a lot of fish do not oviposit as predicted **(Mousa, 1994)**. Mature fish of *L. ramada* may react to stress caused by maturation of the ovaries, adaptation to seawater, and spawning induction **(Mousa & Mousa, 2006)**. Stress has a negative impact on fish growth, reproduction, and osmoregulation **(Wendelaar**

Bonga, 1997; Flik & Wendelaar Bonga, 2001). Additionally, it has a major detrimental impact on the success of reproduction **(Campbell *et al*., 1992; Schreck *et al*., 2001)**.

It is crucial to comprehend the physiological makeup of fish during the cycle of reproduction, acclimation to seawater, and spawning induction for the purpose of identifying possible reasons for fish death before oviposition and to create effective hatchery technology. The mullet's physiology during the cycle of reproduction and the stimulation of spawning, however, is not well understood. Nevertheless, no research has measured the thyroid hormones' daily rhythm in mullet. The circadian pattern of mullet hormones have not been thoroughly examined. Comprehending the daily schedule of the mentioned hormones would provide biologists and other researchers with an initial step to monitor and evaluate the metabolic status and general health of mullet in captivity or wild.

MATERIALS AND METHODS

Broodstock farming

This work was conducted at both El-Serw and El-Matareyya Research Stations between the 1st of January 2022 and the 30th of January 2023. *Liza ramada* fingerlings were stocked in fresh water earthen ponds at El-Serw for one year. In the next year, fish were collected from El-Serw ponds and stocked in both saline groundwater (30‰) and freshwater cement ponds at El-Matareyya **(Mousa *et al*., 2022)**. The obtained mature fish, at least two-years-old, had weights varying from 250 to 460g and an average standard length ranging from 27 to 31.5cm. These fish were obtained alive on a monthly basis around the year. However, to make certain that all specimens were at the maturing phases, during the spawning season (November to January), the fish were obtained half monthly.

Sampling and analytical procedures

During acclimatization to seawater and spawning induction, ten fish from each maturity stage were sampled for blood examination by caudal severance. The chosen fish were narcotized with a solution containing 40mg/ l clove oil (Sigma) during handling **(Mousa, 2010)**. Blood was taken into centrifuge sampling tubes, and the serum was separated with the centrifuge and kept in freezer at –20°C until analysis.

Serum total thyroxine (T4) **(Schurrs & van Weeman, 1977)**, total triiodothyronine (T3) **(Walker, 1977)** and cortisol **(Barry *et al*., 1993)** were measured using the assay for enzyme-linked immunosorbents (ELISA). Glucose and the different blood biochemical parameters were measured by the routine automated biochemistry using the auto analyzer Synchron CX7 clinical system (Bechman Instruments Inco. USA).

Immunoassay procedure

GH and PRL concentrations were calculated utilizing electrochemiluminescence (Roche Diagnostics, Mannheim, Germany; Elecsys 2010) utilizing the subsequent

Elecsys Kits. The microplate reader was employed to acquire the hormone assay with 450nm setting.

Kits for hormonal ELISA

Various kits were utilized to determine hormone levels as:

1- Elecsys prolactin (Catalog Number: 03203093190).

2- Elecsys GH (Number of Catalog: 5390125).

Statistical analysis

ANOVA was employed for data analysis using a randomized block design, with the experiment serving as the blocking factor. The test of honestly significant difference (HSD) from Tukey was employed as the foundation for post hoc comparisons. With Statistical Package for the Social Sciences; SPSS (IBM version 22), all statistical tests were completed. It was statistically regarded significant at $P < 0.05$.

RESULTS

The obtained levels of hormones involved in growth, acclimatization, and response to stress, adding to glucose, total protein and albumin of mature *L. ramada* females represented a circadian pattern (Table 1).

Growth hormone

Growth hormone level demonstrated a difference with time changes both throughout the day and at night. The GH concentrations were at their highest at 6AM (2.8ng/ ml) for freshwater fish and at 12 PM (2.4ng/ ml) for saline water fish (Table 1). While, the minimum level of GH was recorded at 6 PM for freshwater (0.8ng/ ml) and saline water (0.5ng/ ml) fish, as mentioned in Table (1). The concentrations of total protein and albumin recorded a trend comparable to the GH level at the mentioned times (Table 1).

Stress-response and acclimatization hormones

During the times chosen for the study, both day and night, the stress-response hormone cortisol and the acclimatization hormone prolactin exhibited different levels (Table 1). These hormones gave the highest rates at 6AM and 12PM for freshwater (319.2ng/ ml; 3.5ng/ ml) and saline water (224.2ng/ ml; 2.5ng/ ml) fish, respectively. However, the mentioned hormones gave the lowest concentrations at 6PM for the fish stocked in freshwater (142.7ng/ ml; 1.2ng/ ml) and saline water (18.4ng/ ml; 0.8ng/ ml) (Table 1). Glucose concentrations exhibited a similar pattern to cortisol and prolactin at the chosen times for both freshwater and saline water fishes, as indicated from data in Table (1).

Thyroid hormones

The resulting thyroxine level indicated a change during the timings chosen in the present research, as demonstrated in Table (1). Highest concentration of thyroxine was found at 6AM for freshwater fish (93ng/ ml) and at 12 PM for saline water fish (23ng/ ml). While, the lowest value was recorded at 6PM for fish in both waters (14ng/ ml; 6ng/

ml), as presented in Table (1). The value of triiodothyronine did not exhibit a difference during the selected times in fresh water (6.51ng/ ml); while in saline water, it showed a decrease at six in the morning (4.84ng/ ml) and in the evening (4.39ng/ ml), as displayed in Table (1).

Table 1. Physiological changes during the obtaining and handling of mature *Liza ramada* females during spawning season

	Saline water 12 AM*	Saline water 6 AM*	Saline water 12 PM*	Saline water 6 PM*	Fresh water 12 AM*	Fresh water 6 AM*	Fresh water 12 PM*	Fresh water 6 PM*
Cortisol ng/ml	145.6 ± 4.1 [a]	97.4 ± 3.9 [b]	224.2 ± 6.7 [c]	18.4 ± 0.9 [d]	192.3 ± 5.8 [e]	319.2 ± 9.2 [f]	231.9 ± 7.4 [g]	142.7 ± 5.2 [h]
Glucose mg/ml	0.87 ± 0.04 [a]	0.87 ± 0.03 [a]	1.03 ± 0.08 [c]	0.37 ± 0.02 [d]	0.74 ± 0.04 [e]	0.92 ± 0.05 [f]	0.50 ± 0.03 [g]	0.08 ± 0.01 [h]
T_3 ng/ml	6.51 ± 0.21 [a]	4.84 ± 0.14 [b]	6.51 ± 0.20 [a]	4.39 ± 0.19 [d]	6.51 ± 0.22 [e]	6.51 ± 0.23 [e]	6.51 ± 0.20 [e]	6.51 ± 0.24 [e]
T_4 ng/ml	20 ± 1.2 [a]	18 ± 1.1 [b]	23 ± 1.3 [c]	6 ± 0.35 [d]	32 ± 2 [e]	93 ± 3.5 [f]	18 ± 1.2 [g]	14 ± 0.9 [h]
Prolactin ng/ml	1.8 ± 0.15 [a]	1.5 ± 0.13 [b]	2.5 ± 0.23 [c]	0.8 ± 0.07 [d]	1.5 ± 0.12 [e]	3.5 ± 0.33 [f]	1.7 ± 0.17 [g]	1.2 ± 0.11 [h]
GH ng/ml	1.2 ± 0.11 [a]	1.5 ± 0.13 [b]	2.4 ± 0.23 [c]	0.5 ± 0.05 [d]	1.1 ± 0.11 [e]	2.8 ± 0.28 [f]	1.8 ± 0.14 [g]	0.8 ± 0.07 [h]
Total Protein mg/ml	26 ± 1.4 [a]	27 ± 1.4 [a]	33 ± 1.7 [c]	17 ± 0.9 [d]	39 ± 1.9 [e]	42 ± 2.1 [f]	41 ± 2.2 [f]	34 ± 1.8 [h]
Albumin mg/ml	11 ± 0.7 [a]	12 ± 0.6 [a]	13 ± 0.7 [c]	8 ± 0.5 [d]	14 ± 0.9 [e]	17 ± 1.1 [f]	16 ± 0.9 [f]	13 ± 0.8 [h]
Albumin/ Globulin Ratio	0.7 ± 0.08 [a]	0.7 ± 0.06 [a]	0.7 ± 0.07 [a]	0.7 ± 0.06 [a]	0.6 ± 0.05 [e]	0.6 ± 0.04 [e]	0.6 ± 0.05 [e]	0.6 ± 0.07 [e]

* Sampling time: 12AM: 12 Midnight; 6AM: 6 in the morning; 12PM: 12 Midday; 6PM: 6 Evening.
Data are reported as means ± SD.
Significantly different means (P< 0.05) are indicated by different letters (Tukey test).

DISCUSSION

In light of the study's findings, growth hormone, thyroxine, triiodothyronine, prolactin, cortisol, total protein, and albumin levels were noticeably greater with maximal values observed at 12PM and 6AM in mature *L. ramada* females raised in both saline and fresh water, respectively. The diurnal trend of the aforementioned hormones suggests that water salinity and daylight may act as environmental cues regarding the control of hormone circadian rhythm **(Costa *et al*., 2019; Sánchez-Vázquez *et al*., 2019; Zhang *et al*., 2024)**. The recorded levels were suitable for the successful acquiring and stimulation of spawning of *L. ramada* in captivity. Higher levels of the aforementioned hormones enabled the mullet to undergo the physiological changes required for spawning-related acclimatization, stress response, and full maturation **(Martemyanov, 2015; Birnie-Gauvin *et al*., 2023)**.

The mullet had a greater need for homeostasis in the spawning induction because of the stressful conditions, which necessitated constant cortisol production all day long. The obtained values of cortisol levels in mature *L. ramada* females represented a circadian pattern, showing a difference with time changes during night and day. The principal hormone of fish under stress is cortisol. While energy repartitioning during stress is cortisol's main function in helping animals metabolically withstand stressor insult **(Milla *et al*., 2009; Pankhurst, 2016)**, it is generally believed to have an inhibiting effect on reproduction **(Milla *et al*., 2009; Mileva *et al*., 2011; Pankhurst, 2016; Faught & Vijayan, 2018)**. Whereas, chronic cortisol- treatment causes suppression of LH-secreting cells activity and blocks progression of vitellogenic follicular development process in *O. mossambicus* **(Chabbi & Ganesh, 2017)**. Furthermore, increased amounts of cortisol might affect oocyte development, which could lower the embryo's viability **(Mileva *et al*., 2011; Medeiros *et al*., 2016)**.

Growth hormone (GH) has a variety of biological effects, including social behavior, gonadal development, growth, energy mobilization, and appetite **(Le Gac *et al*., 1993; Munro & Lam, 1996; Canosa *et al*., 2007)**. Growth hormone, which is similar to prolactin, helps several teleost fish in seawater acclimation **(Sakamoto & McCormick, 2006; Ferreira-Martins *et al*., 2023)**. According to reports, the chloride cell differentiation and development of the seawater kind and branchial epithelia is managed by cortisol and GH; whereas in freshwater, prolactin and cortisol help develop and differentiate the chloride cells **(Sakamoto & McCormick, 2006)**. Acclimating fish had effects on metabolic and growth parameters, and presumably suppressed their growth potential owing to the prospective energy cost or stress for osmoregulation **(Hayashi *et al*., 2021)**.

Prolactin in fish possesses an extensive array of functions in fish physiology. The prolactin in fish has different actions on reproduction, migration, reproductive development and cycling, brood care behavior, pregnancy, and nutrient provisioning to the young **(Freeman *et al*., 2000; Saha *et al*., 2021)**. PRL is a regulatory hormone for ion and water transport. In bony fish, PRL enhances calcium transport in the gills of both freshwater and euryhaline species, leading to hypercalcemia **(Flik *et al*., 1994; Freeman *et al*., 2000; Anderson & Itallie, 2009; Saha *et al*., 2021)**. Prolactin, a major osmoregulatory hormone in fish has evolved into a hormone that regulates lactation in mammals **(Breves *et al*., 2020)**. In fact, both of these functions require the action of prolactin on epithelial cells and their proliferation **(Freeman *et al*., 2000; Sakamoto & McCormick, 2006)**. Furthermore, PRL assists in the reproductive cycle in addition to the sexual organ growth in fish **(Saha *et al*., 2021)**.

The obtained levels of thyroxine of mature *L. ramada* females represented a circadian pattern; showing a difference with time changes during night and day, and with maximum values observed at 12PM and in morning at 6 o'clock for females raised in both saline and fresh water, respectively. Thyroid axis components respond to environmental signals and go through seasonal and circadian cycles, according to several studies on fish **(Grau, 1988; Cowan *et al*., 2017)**. Some fish species, such as the goldfish, red drum, Atlantic salmon, and winter flounder, have been observed to exhibit circadian cycles of thyroid hormones **(Spieler & Noeske, 1979; Leiner *et al*., 2000; Ebbesson *et al*., 2008)**. The time of thyroid hormones (THs) peak seems to differ byspecies; for example, in the rainbow trout, THs levels are high in males during the day and low at night, and the opposite takes place in females **(Ganzha & Pavlov, 2019)**. THs play numerous functions in teleosts, like feeding and nutrient metabolism, metamorphosis, growth and development, as well as reproduction **(Mousa *et al*., 2018; Deal & Volkoff, 2020; Seale *et al*., 2021; Prazdnikov & Shkil, 2023)**. THs increase the rate of glucose oxidation, and thus increase the amount of metabolic heat produced **(Oki & Atkinson, 2004; Deal & Volkoff, 2020)**.

A comprehensive data on the daily cycle of the hormones would provide an adequate information needed address and maintain the general health and metabolic status of the mullet fish during spawning induction in captivity.

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Conflict of Interest

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

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