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HORMONE STUDY IN FRESHWATER FISH *CHANNA GACHUA* (HAM-1822) DURING PRE-MATURE, MATURE AND SPAWNING BY ENZYME ASSAY BASED CHEMILUMINESCENCE TECHNOLOGY.

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ABSTRACT

The quantity of hormones were observed in premature fish *C.gachua* . A mature fish, with length (15cm) and weight (30 gm) Testosterone has been reported at 0.24 ng/ml in male and 0.04 ng/ml in female, Estradiol 0.004 pg/ml in male and 2.4 pg/ml in female. Progesterone (3.001 ng/ml) in female. DHEAS at 0.2 µg/ml in male and 0.2 µg/ml in female. Lutinizing hormone 0.2 mIU/ml in male and 0.6 mIU/ml in female. Follicle stimulating hormone, (FSH) 32.7 µg/ml in female. Thus, in spawning having length (15cm) and weight (35gm), quantity of testosterone 0.04 ng/ml in, Estradiol in female 0.4 pg/ml, Progesterone 2.01 ng/ml in female. DHEAS 0.002 µg/ml in male and 0.009 µg/ml in female) Lutinizing hormone in male 0.02 mIU/m0 and in female 0.2 IU/ml. Follicle stimulating hormone female 0.01 µg/ml., has been recorded significantly.

KEY WORDS: Hormone, *C.gachua*, Chemiluminescence technology, breeding, Gonads

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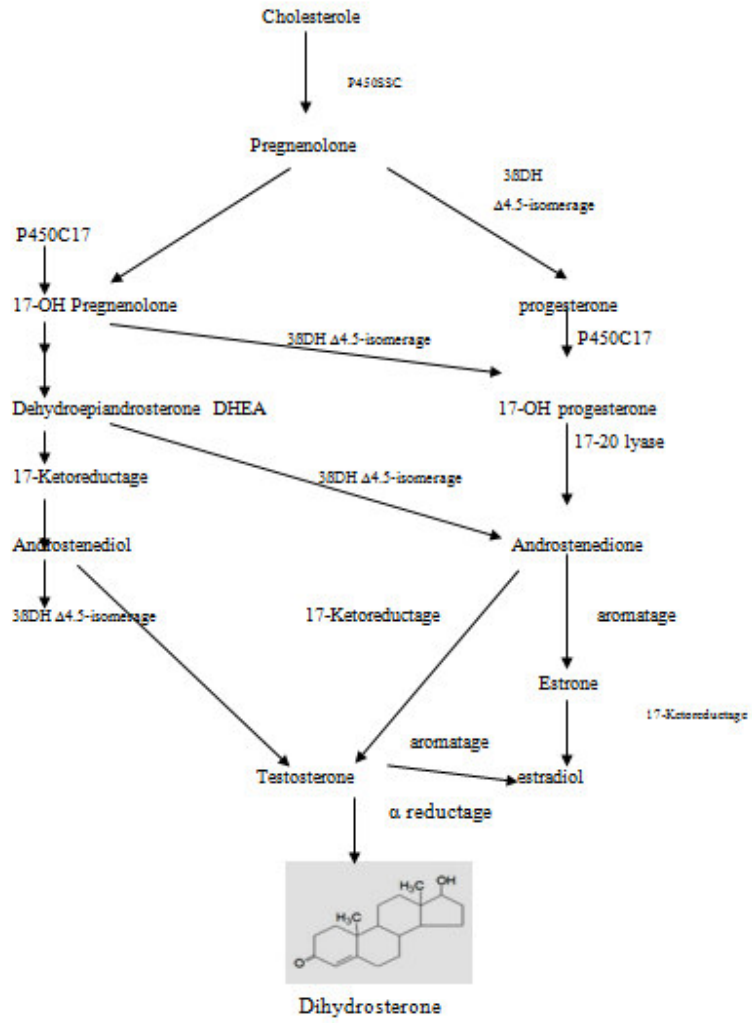
INTRODUCTION

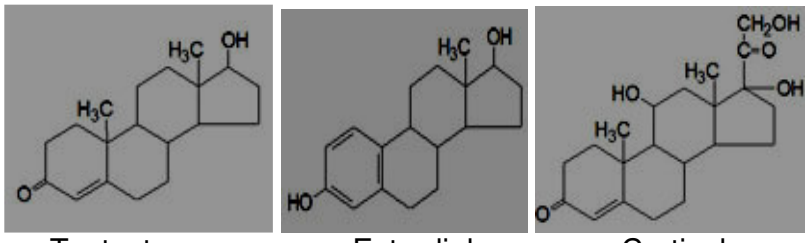
British researchers noticed that in the early 1990s, male fish fed on sewage had testes laden with eggs had turned in to hermaphrodites. Like higher vertebrates, quantity of sex steroids in freshwater fish has been studied by using enzyme assay based Chemiluminescence technology.FSH is a heterodimeric glycoprotein synthesized and secreted by the anterior pituitary gland is involved in the regulation of essential vertebrate reproductive processes such as gametogenesis and follicular growth. Deleterious effects on gonadal development may confirm by a dramatic reduction of the gonadosomatic index due to high concentration of testosterone in female Blázquez. *et. al*. (2001). In addition, around the first year of age, growth was significantly depressed in all groups. The testosterone is the major circulating sex hormone of the male and serves as the prototype for the androgens, the anabolic agents, and androgen antagonists. The endogenous androgens are biosynthesized from cholesterol in various tissues in the body. Majority of the circulating androgens are produced in the testes under the stimulation of the gonadotropin luteinizing hormone (LH). The reduction of testosterone to dihydrotestosterone is necessary for the androgenic actions of testosterone.In many androgen target tissues such as the prostate; the oxidation of testosterone by the enzyme aromatase produces estrogens Brueggemeier (2003). The glycoprotein hormones are structurally and functionally conserved in various vertebrates and have been identified in most lineages of actinopterygian (bony) fish Park *et al*. (2005). Gonadal steroid hormones are produced by the testes and the ovaries, the two most important are testosterone and estradiol. These compounds are under tight biosynthetic control, with short and long negative feedback loops that regulate the secretion of follicle stimulating hormone (FSH) and luteinizing hormone (LH) by the pituitary and gonadotropin releasing hormone (GnRH) by the hypothalamus. Low levels of circulating sex hormone reduce feedback inhibition on GnRH synthesis (the long loop), leading to a elevated FSH and LH. The biosynthetic pathway to sex hormones in male and female gonadal tissue includes the production of the androgens, androstenedione and dehydroepiandrosterone. Testes and ovaries contain an additional enzyme, a 17β-hydroxysteroid dehydrogenase, that enables androgens to be converted to testosterone.Testosterone is one of the anabolic steroidal hormones secreted in large amounts by the testes in males, and to a lesser extent, by the adrenal cortex and ovaries in females. Testosterone is an androgen which has masculinizing effects on individuals.Testosterone exerts its effects right from the perinatal period through puberty and adulthood. It is also related to a variety of social behaviors including aggression, power, sexual behavior, and social dominance. Although females have just about 1/7th the testosterone levels as men, apparently, testosterone still plays a role. In pubescent females, testosterone effects are more subtle but equally important for proper musculo-skeletal development, general anabolic activity, and libido. In both sexes, testosterone enhances aerobic metabolism and increases protein synthesis.Total testosterone: commonly measured using chemiluminescence immunoassay or radioimmunoassay; gold standard is liquid chromatography tandem mass spectrometry (LC-MS). LC-MS is especially helpful in cases of low Testosterone concentration, for example in females and pre-pubertal individuals, as the immunoassays perform poorly at low Testosterone concentrations Wang (2004). Bioavailable testosterone represents biologically active Testosterone, includes both free Testosterone and albumin-bound Testosterone and calculated based on the binding of Testosterone to SHBG and albumin, also, measured directly by the ammonium sulfate precipitation method, which precipitates SHBG and SHBG-bound Testosterone. Free testosterone gold standard is direct measurement by equilibrium dialysis, can also be calculated based on total Testosterone and

SHBG using method validated by Vermeulen (1999). DHEAS, biologically inert steroid produced by the adrenals that becomes active after being converted to androstenedione and then Testosterone in the periphery. Measured with chemiluminescence immunoassay, radioimmunassay, or LC-MS.LH and FSH, workers measured using chemiluminescence immunoassay or radioimmunoassay. Highly sensitive and selective microplate chemiluminescence enzyme immunoassay for the determination of free thyroxine in human serum developed Wang ey al, (2007). Studies were carried out to determine whether the anabolic steroids 17α-methyltestosterone, 11-ketotestosterone, 4-chloro-testosterone acetate, testosterone, oxymetholone and progesterone can promote an increase in body weight when incorporated McBride *et al.* (2009).In males, LH binds to Leydig cells, stimulating production of the principal Leydig cell hormone, testosterone. Testosterone is secreted to the plasma and also carried to Sertoli cells by androgen binding protein (ABP). In Sertoli cells the double bond of testosterone is reduced, producing dihydrotestosterone. Testosterone and dihydrotestosterone are carried in the plasma, and delivered to target tissue, by a specific gonadal-steroid binding globulin (GBG). In a number of target tissues, testosterone can be converted to dihydrotestosterone (DHT). DHT is the most potent of the male steroid hormones, with an activity that is 10 times that of testosterone. Because of its relatively lower potency, testosterone is sometimes considered to be a prohormone.A hormone is a chemical substance. It's secreted by one tissue and travels by way of body fluids to affect another tissue in body. In essence, hormones are "chemical messengers." Many hormones, especially those affecting growth and behavior, are significant to animal. The amount and levels of hormones change daily. The sex hormones, estrogen and testosterone, are secreted in short bursts and pulses.Dehydroxyepineprine **(**DHEA) is a steroid hormone which is structurally similar to other steroid hormones (such as estrogen, progesterone and testosterone), but which possesses its own spectrum of biologic effects. Scientists have known for years that DHEA is secreted by the adrenal gland and that this is a greater quantity of this hormone produces than any other adrenal steroid. In both humans and animals, the decline of DHEA production with aging is associated with immune depression, loss of sleep, decreased feelings of well-being, and increased mortality.Estrogen is an entire class of related hormones. They include estriol, estradiol, and estrone. Estradiol is made from the placenta. It's produced during pregnancy. Estradiol is the primary sex hormone of spawning fishes and also in higher vertebrates. It is formed from developing ovarian follicles. Estradiol is responsible for female characteristics and sexual functioning. Also, estradiol is important to female bone health and condiotion. Estradiol contributes to most gynecologic problems such as endometriosis and fibroids and even female cancers in human beings. Estrone is widespread throughout the body of anima. It is the only one of the estrogens that's present in any amount in female after ovulation. The principles of chemiluminescence and the application of chemiluminescent labels and substrates in immunoassays are reviewed Rongen *et al.* (2010) Normal Testosterone and Estrogen levels in higher vertebrates, it would surprise to know that, male don't have a monopoly on testosterone. Testosterone belongs to a class of male hormones called androgens. But, female also shows testosterone level. The ovary produces both testosterone and estrogen. Relatively small quantity of testosterone is released into bloodstream by the ovaries and adrenal glands. In addition to being produced by the ovary, estrogen is also produced by fat tissue in the body. These sex hormones are involved in the growth, maintenance, and repair of reproductive tissues. But that's not all, they influence other body tissues and bone mass as well.

Schematic representation of synthesis of male sex hormone



Chemical structure of sex hormones



Testosterone Estradiol Cortisol

Testosterone is an androgen, male sex hormone synthesized in the testes, responsible for secondary male sex characteristics, produced from progesterone. Estradiol is an estrogen, principal female sex hormone, produced in the ovary, responsible for secondary female sex characteristics Cortisol is dominant glucocorticoid in humans, synthesized from progesterone in the zona fasciculata of the adrenal cortex, involved in stress adaptation, elevates blood pressure and Na^+ uptake, numerous effects on the immune system

MATERIALS AND METHODS

Live freshwater male and female fishes, during premature, mature and spawning period were collected from Godavari River near Aurangabad. Disposable syringes (2ml) were used to suck the blood from the caudal vein. Serum was obtained using centrifuge matching at 3000 rpm. The red color pigmentations were removed by using activated charcoal (black). Method and apparatus for improved luminescence assays using particle concentration chemiluminescence detection, Massey et al., (2002).

Chemiluminescence technology

Chemiluminescence is the generation of electromagnetic radiation as light by the release of energy from a chemical reaction. Serum was further used for photo-detector in Chemiluminicence. Different tracers and signaling reagents were used to carry out luminescence reactions for hormones.Standardization of chemilumnisence apparatus for detection and quantification of freshwater fish sex hormone from serum. Standardization was done each time for total hormones using standard markers from USA.

Immunoassay

Testosterone (17â-hydroxyandrost-4-ene-3-one) is a C19 steroid with an unsaturated bond between C-4 and C-5, a ketone group in C-3 and a hydroxyl group in the a position at C-17. This steroid hormone has a molecular weight of 288.4. Testosterone is the most important androgen secreted into the blood. In males, testosterone is secreted primarily by the Leydig cells of the testes; in females ca. 50% of circulating testosterone is derived from peripheral conversion of androstenedione, ca. 25% from the ovary and ca. 25% from the adrenal glands. Testosterone is responsible for the development of secondary male sex characteristics and its measurements are helpful in evaluating the hypogonadal states.In higher vertebrate female, high levels of testosterone are generally found in hirsutism and virilization, polycystic ovaries, ovarian tumors, adrenal tumors and adrenal hyperplasia. In men, high levels of testosterone are associated to the hypothalamic pituitary unit diseases, testicular tumors, congenital adrenal hyperplasia and prostate cancer.

Principles of test

The Testosterone Chemiluminescence Immunoassay is based on the principle of competitive binding between Testosterone in the test specimen and Testosterone-HRP conjugate for a constant amount of rabbit anti-Testosterone. In the incubation, goat anti rabbit IgG-coated wells are incubated with 10 µl of Testosterone standards, controls, patient samples, 100 µl Testosterone-HRP conjugate reagent and 50 µl rabbit anti-Testosterone reagent at 37°C for 90 minutes. During the incubation, a fixed amount of HRP-labeled Testosterone competes with the endogenous Testosterone in the standard, sample, or quality control serum for a fixed number of binding sites of the specific Testosterone antibody. Thus, the amount of Testosterone peroxidase conjugate immunologically bound to the well progressively decreases as the concentration of Testosterone in the specimen increases. Unbound Testosterone peroxidase conjugate is then removed and the wells washed. Next, A solution of chemiluminescent substrate is then added and read relative light units (RLU) with a Luminometer. The intensity of the emitting light is proportional to the amount of enzyme present and is inversely related to the amount of unlabeled TESTOSTERONE in the sample. By reference to a series of T Testosterone standards assayed in the same way, the concentration of testosterone in the unknown sample is quantified.

Materials provided with Test Kit

Goat Anti-Rabbit IgG-coated microtiter wells, 96 wells. Testosterone Reference Standards: 0, 0.1, 0.5, 2.0, 6.0 and 18.0 ng/ml. Liquids, 0.50 ml each, ready to use. Rabbit Anti-Testosterone Reagent (pink color), 7.0 ml Testosterone-HRP Conjugate Reagent (blue color), 12 ml 20x Wash Buffer, 30 ml Chemiluminescence Reagent A, 6.0 ml. Chemiluminescence Reagent B, 6.0 ml. Distilled water. Precision

pipettes: 0.01ml, 0.05ml, 0.10ml.Disposable pipette tips.Glass tube or flasks to mix Reagent A and B., Microtiter well luminometer, Vortex mixer or equivalent, Absorbent paper and Graph paper.

Reagents

To prepare substrate solution, make an 1:1 mixing of Reagent A with Reagent B right before use. Mix gently to ensure complete mixing. Discard excess after use. Prepare the washing solution by diluting 1 part of the 20X PBS concentrate to 19 parts of distilled water.

Assay procedure

Secure the desired number of coated wells in the holder. Dispense 10 µl of standards, specimens and controls into appropriate wells. Dispense 100 µl of Testosterone-HRP Conjugate Reagent into each well. Testosterone standards assayed in the same way, the concentration of testosterone in the unknown sample is quantified. Similar test was carried for DHEAS, Testosterone, estradiol, progesterone, cortisol, luteinizing hormone and follicular stimulating hormone.The estimation of hormones can be done with an ultraviolet spectrophotometric method presented for the quantitative estimation of steroids which are in concentrations of more than 1.25 µ/ml. Estradiol-17β, progesterone, and testosterone were used in these studies. Based on uv light absorption at 230 nm for estradiol and progesterone, and at 240 nm for testosterone, the relative concentration of each steroid could be estimated. This method is very simple and rapid. It is economical, requires no sophisticated instruments, and is very practical for estimating steroids in pharmaceutical preparations, chemicals, or biological specimens Khayam H**.** (2004).

RESULTS

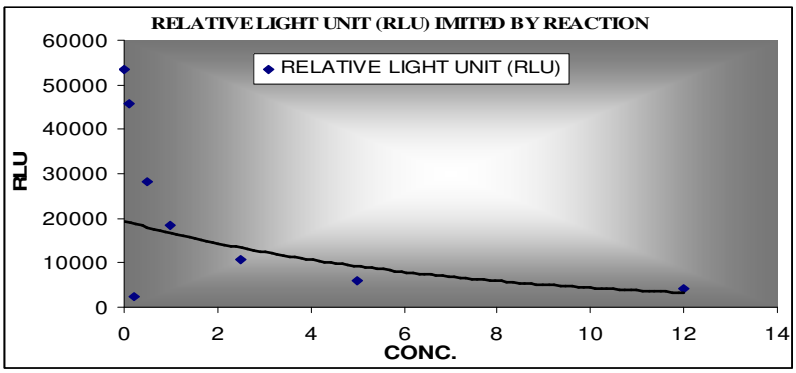
Table1

Standardization of chemilumnisence apparatus for detection and quantification of freshwater fish sex hormone testosterone from serum .

Wells.	ID of Wells	RELATIVE LIGHT UNIT (RLU)	CONCENTRATION
1	C1	53546	0.0
2	C2	45873	0.1
3	C3	28334	0.5
4	C4	18534	1.0
5	C5	10825	2.5
6	C6	5965	5.0
7	C7	4057	12.0
9	1 (SAMPLE)	2450	0.2

Figure 1

relative light unites emitted by antibody (hormone molecule) and antigen (tracer used) in reaction.



Hormones detected and quantified during premature, mature and spawning in *C.gachua*. The growth of freshwater fish *C.gachua* was isometric (table 1), (fig.1) the levels of sex hormones quantity are in very minute tress (table 2 and 3). In premature fish having length (12cm) and weight (30 gm), quantities of testosterone 0.11 ng/ml in male, estradiol 0.24 pg/ml in female, progesterone 3.01 ng/ml in female (dehydroepiandrosterone) DHEAS in male 0.001 µg/ml, 0.006 µg/ml in female.Luteinizing hormone (LH) 0.15 IU/ml in female follicle stimulating hormone (FSH) 0.41 in female. In mature fish having length (15cm) and weight (35gm),, level of testosterone 0.24 ng/ml in male and 0.04 ng/ml in female, estradiol 0.004 pg/ml in male and in female 2.4 pg/ml, progesterone 3.001 ng/ml in female. DHEAS 0.2 µg/ml in male and in female 0.2 µg/ml. Lutinizing hormone, (LH)0.2 IU/ml in male and 0.6 IU/ml in female.Follicle stimulating hormone (FSH) 32.7 µg/ml in female. Thus, in spawning having length (15cm) and weight (35gm), quantity of testosterone 0.04 ng/ml in male and -0.89 ng/ml in female, estradiol in male -0.4 pg/ml and in female 0.4 pg/ml, progesterone in male -2.01 ng/ml and 2.01 ng/ml in female DHEAS 0.002 µg/ml in male and in female 0.009 µg/ml) Lutinizing hormone (LH) in male 0.02 mIU/m0 and in female.0.2 IU/ml l. Follicle stimulating hormone (FSH) -3.87 µg/ml in male and in female 0.01 µg/ml. Has been recorded significantly.

Table 1
Length –weight relationship in** C.gachua **(Ham-1822)

Month	Mean Length of fish	Mean Weight of fish					
	ln (cm)	ln (gm)	X2	Y2	XY	Mean □SD Weight	Correlation value
	X	Y					
Jan.	13.9	12.9	193.21	166	179.31	0.4801	0.78
Feb.	12.5	18.5	156.25	342	231.25	0.1829	0.784
Mar.	12.3	20	176.89	400	266	0.8853	0.889
April	12.2	22	148.84	250	192.76	0.8897	0.786
May	12.1	30	198.81	484	310.2	0.6697	0.775
June	13.9	30	193.21	166	179.31	0.889	0.897
July	12.5	30	156.25	342	231.25	0.998	0.878
Aug.	12.3	20	176.89	400	266	0.664	0.75
Sep.	12.2	15.8	148.84	250	192.76	0.1018	0.784
Oct.	14.1	22	198.81	484	310.2	0.331	0.88
Nov.	12.2	15.8	148.84	250	192.76	0.333	0.98
Dec.	12.1	22	198.81	484	310.2	0.454	0.999

Figure 2
Shows growth in Channa gachua. (Ham* 1822*) during (2010)

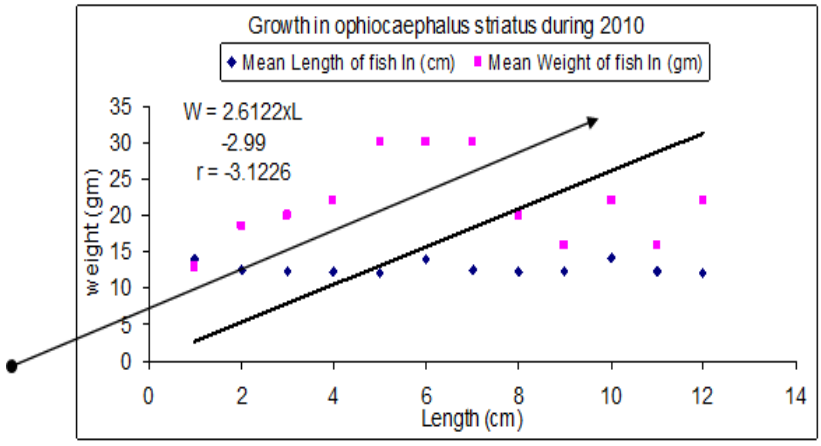


Table 2
Hormones detected and quantified during premature, mature and spawning in male Channa gachua. (Ham* 1822*)

Hormones	Premature	Mature	Spawning
DHEAS	0.001 µg/ml, (±0.17) *	0.2 µg/ml (±o.12)**	0.002 µg/ml (±0.11)*
Testosterone	0.11 ng/ml (±0.13)*	0.24 ng/ml (±0.34)**	0.04 ng/ml (±0.23)*
Estradiol	NA	0.004 pg/ml (±0.53)*	NA
Progesterone	NA	NA	NA
Coratisole	NA	NA	NA
Lutinizing hormone (lh)	NA	NA	NA
Folicle stimulating hormone(fsh)	NA	NA	NA

**** Significant ** moderate ***highly significant***
NA indicates that there is no reactivity of tracers in serum so no hormone present.

Table 3
Hormones detected and quantified during premature, mature and spawning in female Channa gachua. (Ham* 1822*)

Hormones	Premature	Mature	Spawning
DHEAS	0.006 µg/ml (±0.68)*	0.2 µg/ml (±0.78)**	0.009 µg/ml (±6.75)*
Testosterone	NA	0.04 ng/ml (±0.69)*	NA
Estradiol	0.24 pg/ml (±0.68)*	2.4 pg/ml (±0.85)**	0.4 pg/ml (±0.60)*
Progesterone	3.01 ng/ml (±0.92)**	NA	2.01 ng/ml (±0.74)*
Cortisole	NA	NA	NA
Luteinizing hormone	0.15 mIU/ml (±0.75)*	0.6 mIU/ml (±0.66)**	0.2 mIU/ml (±0.76)*
Folicle stimulating hormone	0.41 µg/ml (±0.64)*	32.7 µg/ml (±0.99)***	0.01 µg/ml (±0.68)*

**** Significant ** moderate ***highly significant***
NA indicates that there is no reactivity of tracers in serum so no hormone present.

DISCUSSION

The advanced and sensitive chemilumnicence technology is useful to trace out minute quantity of biomolecules, i.e. antibody-antigen bonding which liberates photons and trapped by photodetecter and relative light unit (RLU) recorded in CLIA. The specific functions of FSH in fish are not yet clearly understood. In this study, we show that FSH increases the secretion rates of estradiol E_2 and testosterone 11-KT in females and males, respectively. This similar findings in other species; FSH and LH of salmon have been found to be equally present in stimulating estradiol E_2 secretion from the vitellogenic ovary of *amago* and *coho* salmon, but FSH was less potent in stimulating secretion of 17α, 20β, dihydroxy-4-pregnen-3–1 from post-vitellogenic oocytes Suzuki K. (1988); Swanson P. (1991).There is a growing body of evidence on the regulation and patterns of gonadotropin gene expression (reviewed by Yaron *et al*. (2001). Our understanding of the unique biological functions of the two gonadotropins in fish is still incomplete, primarily because of a lack of purified hormones, particularly FSH. We demonstrate the production of a biologically active FSH and its use as a tool for revealing the biological relevance of FSH in *C.gachua* in serum. In other study Methyltestosterone (MT) enhanced the chemiluminescence of potassium permanganate sodium thiosulphate system in sulphuric acid medium, and this was used as the basis of a novel flow-injection chemiluminescence method for the determination of methyltesterone. Xie *et al* . (2005) FSH also increased estradiol E_2 levels in common carp.Atlantic *halibu*t Weltzien *et al.* (2003), and *Japanese eel* Wong *et al.* (2006). FSH has been reported to stimulate the incorporation of vitellogenin into the ovaries of rainbow trout, which is cooperated by the surge of FSH concomitant with the new generation of

vitellogenic oocytes in trout and in tilapia Levavi *et al*., (2006).

CONCLUSION

Like other vertebrates, fishes also produce hormones and released in blood. Lutinizing hormone and follicular stimulating hormones found at peak during mature period but progesterone and estradiol are vice-versa level in spawning, there was low level of progesterone as compared to estradiol in female fish. During study, testosterone which synthesized by gonad cell was detected along with progesterone precursors produced by follicular cell and estradiol in vice-versa level. All hormones are present significant quantity in matured fish.

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