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Research Article

Presence of Neurosteroids and Expression of Neuropeptides in the Brain of Indian Salmon, *Eleutheronema tetradactylum*

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ABSTRACT

The Indian Salmon (*Eleutheronema tetradactylum*) is one of the most commercially significant species with high nutritional value. This species of fish was abundantly available in India during the early 1950s but it has been fast declining in the Indian coast in the recent years due to over exploitation and overfishing. Besides, there is very limited genomic information available on this species. The present study encompasses brain steroid profile using HPLC (high-performance liquid chromatography) and gene expression analysis of peptides such as leptin receptor and insulin receptor-a in the brain of the fish using RT-PCR (Real Time-Polymerase Chain Reaction). The presence of different concentrations of steroids such as progesterone and pregnenolone were recorded in the whole brain of immature and vitellogenic female samples. On the other hand, these hormones as well as 17α-pregnenolone were detected in various regions of brain. Expression of insulin receptor-a was noticed in the female brain and not in the male brain at the immature reproductive stage. Leptin receptor was found to be expressed both in the female and male brain samples. Although the different levels of steroids and the expression of peptide receptors were found in the brain of Indian Salmon, the interrelation between the steroids and peptides were not elucidated from this study. Subsequent studies using Next Generation Sequencing (NGS) will provide a better understanding of this fish. Further, this can aid in promoting culture practices and induced breeding techniques of *E. tetradactylum*. In the long run, this can assist in bringing back the population of this fish in Indian waters and strengthen its importance as a fishery resource.

Keywords: Indian Salmon; Neurosteroids; Neuropeptides

INTRODUCTION

Eleutheronema tetradactylum is one of the most promising pelagic fish species and is considered to be an important aquaculture resource. It prefers shallow turbid water, soft substrates and is found in a variety of near-shore habitats. It is a protandrous hermaphrodite that turns into female after 2 years with a maximum lifespan of approximately 7 years growing up to a length of 1 meter or more [1]. In the early 1950s, this was found to be progressively increasing in large numbers in the Indian waters. Statistics revealed that the threadfin ranked sixth with respect to exports, the Indian salmon being the common species obtained in three different sizes [2]. In the recent times, the predominance of this species has reduced drastically due to overexploitation. Also, the amount of genomic information on this species is very limited.

Brain is a commanding site for synthesis of reproductive hormones such as estrogen, testosterone, pregnenolone and progesterone. There has been significant work done to explain the diverse functions of these hormones in influencing the reproductive growth [3]. Brain neurosteroids and neuropeptides play a major role in the induction and regulation of the synthesis of hypothalamic GnRH secretion. Neuropeptides exert several different biological effects within the brain such as the regulation of gene transcription, local blood flow, synaptogenesis, and glial cell architecture [4]. Besides, neuropeptides are also responsible for the onset of many physiological processes. As the target for action of sex steroids, neuropeptides serve as molecular motifs that control reproduction. Hence, the study was aimed at identifying and quantifying the various steroids present in the different regions of the brain and whole brain of *E. tetradactylum*. Also, the gene expression of neuropeptides such as leptin receptor and insulin receptor-a were studied using qPCR in the male and female brain of *E. tetradactylum*.

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MATERIALS AND METHODS

Sample collection

Samples of *E. tetradactylum* were collected from Kovalam (12° 47' 14.3"N 80° 15' 03.4"E) and Kalpakkam (12° 30' 37.6"N 80° 09' 43.4"E) near Chennai, Tamil Nadu during the month of February 2018 (at the previtellogenic stage) and at the end of March 2018 (at the vitellogenic stage) and were transported to the laboratory to do further analysis. The live samples were maintained in tanks for a stipulated time period and were fed regularly. Later, they were anesthetised and their brains were dissected out for further analysis.

Analysis of Neurosteroids

Preparation of sample: The whole brain of Indian Salmon was removed and divided into four regions as R1, R2, R3 and R4 (Figure 1 and Table 1) coalesced together from female fishes and also total brain samples were separated out, weighed and stored in 0.6% saline solution. Each of the samples was homogenized with saline solution. Samples were pooled together and processed further to study the HPLC steroid analysis.

Extraction of steroids: Steroids were extracted from tissues by using cyclohexane and ethyl-acetate in 1:1 ratio. This extract was then vortexed and allowed to stand until a clear supernatant was obtained. The supernatant was pipetted out and transferred to a clean test tube. The above step was repeated thrice in order to obtain all the steroids present in the brain tissue samples. The test tubes containing the steroids were left undisturbed at room temperature for solvent evaporation. The residues were redissolved in ethanol and used for HPLC detection.

High-Performance Liquid Chromatography (HPLC): The neurosteroids present in the brain of *E. tetradactylum* were identified and separated using HPLC (Shimadzu VP10). The reverse phase C18 column (ODS) with bonded silica was used for separation. The mobile solvent phase used was acetonitrile and water in the ratio 40:60. The sample was injected into 20 µL loop. The column worked optimally at room temperature. The separation occurred at a flow rate of 0.8 mL/minute in binary gradient mode with 40 minutes program. The results were obtained using a UV-Vis detector set to read at 244 (λ max) and PDA detector set to read at 190 nm and 240 nm. The standard reference steroids were chosen to be estradiol-17β; testosterone; pregnenolone; 17α-pregnenolone; progesterone; 17α-hydroxyprogesterone; 20β-hydroxyprogesterone; 17α, 20β dihydroxyprogesterone; and 17α, 20β-dihydroxyprogesterone. Table 2 shows the retention time (RT) value, max λ and the area covered in each standard steroids of 20 µg.

Gene expression studies

Total RNA extraction: Total RNA was isolated from the total brain tissue using TRIzol method. The RNA was loaded for agarose gel electrophoresis and then quantified using UV-Spectrophotometer.

cDNA synthesis: The process of cDNA synthesis was done using the GCC Biotech cDNA Synthesis Kit (M-MLV Reverse Transcriptase, 10x M-MLV RT Buffer, oligo-dT primer, dNTP, ribonuclease inhibitor) with the help of a thermocycler.

Primers: Neuropeptides chosen for the gene expression studies were leptin receptor and insulin receptor-a. With the references acquired from the literature, it was verified that the insulin receptor-a and leptin receptor were significantly expressed in the brains of many teleost fishes. However, due to the lack of genomic information of Indian Salmon, the suitable primer was constructed with the available nucleotide sequence in NCBI bank. Therefore, the primers of these peptides used for the experiment are given below in the Table 3.

RT-PCR and gel electrophoresis: The process of qPCR was done using the Bio-Rad RT-PCR instrument and Bio-Rad Sybr Green Master Mix Kit. The initial denaturation step was at 94°C for 3 minutes and denaturation was set at 94°C for 10 seconds, then annealing was at 57°C for 30 seconds and 39 cycles were carried out. Later, 0.8% gel was casted and bands were observed.

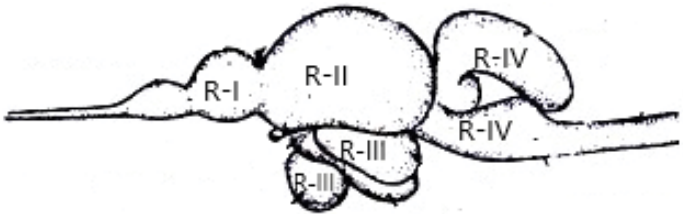

Figure 1: Brain of the Indian Salmon, *E. tetradactylum* with divisions into four regions for the present study.

Table 1: Regions of brain of Indian Salmon, *E. tetradactylum*.

Region 1 (R-I)	Olfactory bulb, dorsal and ventral nuclei of telencephalic area, anterior part of parvocellular preoptic nucleus.
Region 2 (R-II)	Posterior part of parvocellular preoptic nucleus, habenula, optic tectum, torus longitudinalis.
Region 3 (R-III)	Ventral and caudal zone of periventricular hypothalamus.
Region 4 (R-IV)	Corpus cerebelli, oculomotor nucleus, valvula cerebelli, lobus caudalis cerebelli, corpus mamillare, crista cerebellaris, octaval nucleus, facial lobe.

Table 2: List of standards and their respective values of retention time, wavelength and peak area used for HPLC analysis of brain samples of Indian Salmon, *E. tetradactylum*.

Reference standard steroids (20 µL @ 1 µg/µL)	Retention Time (min)	λ max (nm)	Peak Area
Estradiol-17β (E2)	8.18	205, 279	63740830
Testosterone (T)	9.31	242	74713375
Pregnenolone (Preg)	30.10	197	27794628
17α-Pregnenolone (17α-Preg)	10.08	194	25272997
Progesterone (P)	26.00	242	62317092
17α- hydroxyprogesterone (17α-P)	11.43	244	28583889
20β-hydroxyprogesterone (20β-P)	27.26	244	40900467
17α, 20β-dihydroxyprogesterone (17α, 20β-P)	8.56	244	48225488
17α, 20β-dihydroxyprogest-erone (17α, 20β-P)	9.63	244	37116508

RESULTS

The brain regions of I, II, III and IV were the pooled samples of brain taken from the fishes having vitellogenic stages of ovary. Table 4 data shows that pregnenolone, 17α-pregnenolone and Progesterone were found to be present in all regions of the brain. Besides, estradiol-17β was detected in R1 and R3 and 17α-hydroxyprogesterone was found in R2 only. Both previtellogenc female and vitellogenic

female brain samples were found to have pregnenolone and progesterone whereas 17α-pregnenolone was detected only in the vitellogenic female. Estradiol-17β and 17α-hydroxyprogesterone were found to be in the non- detectable ranges (Table 5).

With all the obtained results (Figure 2), it can be inferred that the leptin receptor was significantly expressed in the brains of both male and female Indian Salmon. Alongside, insulin receptor-a was found only in the female brain sample and not in the male brain.

DISCUSSION

This is the first demonstration of neurosteroids and neuropeptides done in *E. tetradactylum*. It was found that the neurosteroids have varied distributions in the different regions of the brain and total brain with respect to their concentrations. Particularly, pregnenolone, progesterone and 17α-pregnenolone were found in all the brain regions. Concentrations of pregnenolone and 17α-pregnenolone were the highest in the cerebral region and that of Progesterone was the maximum in region R-II. Besides, some concentration of estradiol was found in brain regions R-I and R-III along with 17α-hydroxyprogesterone in region R-II alone. The same pattern was seen in the whole brains as well. It can be hypothesized that such locally produced steroids impact reproduction and sexual behaviour similar to that of what happens in higher vertebrates [5]. Also, it could be possible that locally produced steroids in teleost fishes operate at synaptic and dendritic spine plasticity in order to regulate behaviour and/or also brain sex changes [6].

Although many peptides are involved in the interplay among appetite, metabolic rate, and energy stores, leptin is arguably the best understood candidate for a central regulating system [7,8]. In fishes such as sunfish, rainbow trout, largemouth bass, channel

Table 3: Primers and their sequences used for gene expression studies in Indian Salmon, *E. tetradactylum*.

Primer	Sequence
Leptin Receptor	leprFWD - 5' CTCCAGTGACGAAGGCAACTT 3'
	leprREV - 5' GGGAAGGAGCCGGAAATGT 3'
Insulin Receptor-a	insraFWD - 5' GGAGCCCACTCGTCTAACAAA 3'
	insraREV - 5' CGCCGTTGTGAATGACGTATTC 3'

Table 4: Presence of steroids in the regions of the brain of Indian Salmon, *E. tetradactylum*.

Steroids identified (in µg/g)	Brain Region I	Brain Region II	Brain Region III	Brain Region IV
Estradiol-17β	1.72	nd	0.05	nd
Pregnenolone	0.22	0.15	0.01	0.90
17α-Pregnenolone	10.42	2.25	0.15	7.37
Progesterone	5.65	13.22	1.77	5.95
17α-Progesterone	nd	0.9	nd	nd
Nd: Non detectable range.				
Values represent the mean value of three repeated run of HPLC.				

Table 5: Presence of steroids in the whole brain of Indian Salmon, *E. tetradactylum* at two different period of female reproduction.

Steroids identified	Whole Brain of previtellogenic female	Whole Brain of vitellogenic female
17β-Estradiol	nd	nd
Pregnenolone	0.55	11.40
17α-Pregnenolone	nd	3.20
Progesterone	6.65	3.55
17α-Progesterone	nd	nd
nd - Non detectable range. Values represent the mean value of three repeated run of HPLC.		

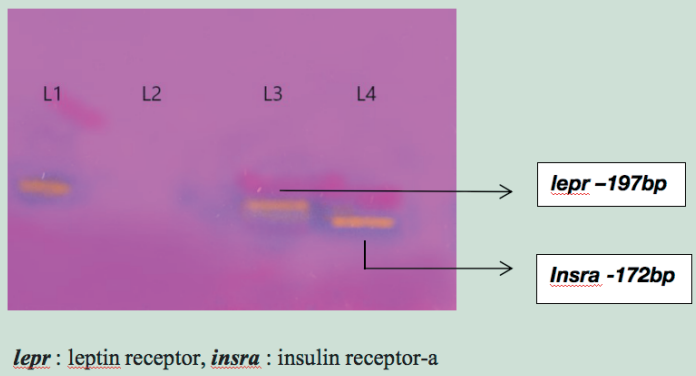

Figure 2: Real time PCR product of the peptides found in brain samples of Indian Salmon, *E. tetradactylum* observed through an UV-Illuminator (Lanes of L1 to L4 are leptin receptor of male brain, insulin receptor-a of male brain, leptin receptor of female brain and insulin receptor-a of female brain, respectively).

catfish and white crappie, [9] suggested a hypothesis to justify large concentrations of leptin and its receptor in brain. Leptin receptor is found to protect leptin from proteolysis. On the same lines, leptin receptor was found to be expressed in both the male and female brain of *E. tetradactylum*.

Insulin is said to be a well characterised peptide hormone which has been identified in most vertebrates. It was also found that the binding of insulin to its receptor occurred in a systematic way and the functional properties of the insulin receptor in different species over a period of evolution remained well conserved [10]. Duguay et al. [11] detected three forms of IGF-I mRNA for coho salmon and these three mRNA forms are equivalent to rainbow trout IGF-I: Ea-1, Ea-3, and Ea-4. By using the same approach, Wallis and Devlin [12] have also detected three size forms of IGF-I mRNA for Chinook salmon. These three size forms correspond to rainbow trout IGF-I: Ea-4, Ea-2, and Ea-l [13]. In the present study, insulin receptor-a was found to be expressed in the female brain. This may be due to higher concentration of insulin receptor-a in female brain compared to the male brain which might have had trace quantities.

CONCLUSION

The results of this study provided an insight about the neurosteroids and neuropeptides found in the fish. However, we were not able to arrive at the interrelation between the steroids and the peptides identified. This in turn paves way for future studies to be carried out using Next Generation Sequencing (NGS) technologies to understand better the genetic make-up of this organism. Therefore, in a broader perspective, this study can facilitate improved culture practices, induced breeding techniques, sexual modulation of this species. In the long run, this can assist in bringing back the population of this fish in Indian waters and strengthen its importance as a fishery resource.

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