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Exploring Molecular Mechanisms of 'GONODOTROPIN RELEASING HORMONE-1' (GNRH-1) in an Animal Model (*CATLA CATLA*)

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Abstract

Hormone-induced breeding of fish has been effectively achieved with Gonopro-FH, a salmon GnRH-1 analog, in several teleost fish but not quite successfully in the *Catla catla.* In order to analyze the rationale behind GnRH-1 ineffectiveness in this species, we investigated the effect of Gonopro-FH injection on the GnRH receptors of the ovary and other extra-pituitary organs, since GnRH receptors are known to be over-expressed during spawning. RT-PCR analysis revealed that the GnRH receptor-1 is present not only in the ovary, but also in the liver, brain and heart tissues of the *Catla catla*. However, the expression of the receptor declined in a time-dependent manner in response to Gonopro-FH injection in all the above tissues. Concomitant changes were observed in the gene expression of the ovary, liver, brain and heart tissues.

Keywords: *Catla catla*, Gonopro-FH, GnRH-1, Ovary, Spawning

Introduction

Production of animals for the aquarium hobbyist trade is rapidly growing sector of aquaculture industry, and it will continue to become more important as restrictions are placed on collected animals from the wild (Lethimonier *et al.,* 2004). Most of the aquacultural production of ornamental fish focuses on freshwater species. Approximately 90% of freshwater ornamental

fish are captively bred (Servili *et al.,* 2010). The fish also occurs in school at the surface in small high-gradient upland stream (Moles *et al.* 2007). When a species is discovered by the aquarium trade, the sudden interest often leads to decline in wild populations (Lethimonier *et al.,* 2004). Thus for sustainable utilization of the fishes, captive breeding is essential. Thus the objective of the research is to study the induced breeding technique by oral delivery of Gonopro-FH a synthetic gonadotropin releasing hormone analogue (SGnRH).

In order to ensure a reliable supply of quality fish seed, various techniques have been developed for breeding pond fish under controlled conditions. Where some methods require only elementary changes in environmental conditions, others require sexual segregation of mature specimens or substrates for attachment of eggs. The most sophisticated and efficient technique till date is the use of hormones to induce spawning (Staff, 1983, Richardson, 1988). Although the technique of induced spawning by hypophysation of cultivated fishes is still in its early stage of development, it has achieved various degrees of success in different countries and for different species of fish.

The major aim of this work is to investigate the role of GnRH on the reproductive axis and, in particular, the relationship between the reproductive and GnRH systems. For this purpose, we have analysed the daily variations in the ovary expression of different GnRH forms and GnRH receptors present in the *Catla catla* by gene expression analysis by quantitative PCR.

Materials and Methods

Animal model

Healthy and adult specimens of *Catla catla,* weighing 350 ± 15 gm, body length of 20–24 cm, were collected from the Kollathur lake, Chennai. Fish were acclimatized to laboratory conditions and reared according to the Animal Care guidelines of Bharathidasan University. Adult ovulatory female fish were used for the experiments. Water temperature varied according to the surrounding environmental temperature. Three fish were used for each dose or time point mentioned in the subsequent experiments for statistically significant data.

Gonopro-FH administration

Gonopro- FH was obtained from Amrit Pharmaceuticals Ltd., India, as a ready-to-use liquid in a standard dose. The volume injected was calculated by multiplying the weight of the fish by 0.4 ml/kg, the amount recommended by the manufacturers for successful induced spawning of carps and other fish. Gonopro- FH was injected intramuscularly at the base of the dorsal fin in females. The control animals were sham injected. The treatment was given at two different intervals (5 hrs and 10 hrs). At the end of the experiment, fish were anesthetized with an overdose of chloroform and sacrificed at 5 hours and 10 hours post injection. The relative GnRH receptor-II mRNA expression levels in different tissues (Ovary, Liver, Brain, Heart) as a result of Gonopro- FH injection were assessed at 5 hours and 10 hours, based on the pulsatile nature of GnRH release and episodic secretion of gonadotropic hormones from the anterior pituitary gland in order to control reproductive functions (Krsmanovic *et al.,* 2003). Tissues were harvested from the Phosphate Buffer Saline (PBS) injected (control) and Gonopro- FH injected (treated) groups.

RNA isolation

Ovary, liver, brain and heart tissues were collected from the ovaprim-injected and control fish. 100 mg of tissue samples were frozen quickly in liquid nitrogen and total RNA was isolated using TRI reagent (SIGMA) following manufacturer's instructions.

cDNA Synthesis

RNA is reverse transcribed into single-stranded cDNA, which can be used directly for subsequent PCR amplification with gene-specific primers in conventional thermal cyclers and real-time PCR instruments or for other downstream applications.

Conversion of RNA to cDNA

Reverse Transcriptase is an enzyme which has RNA-directed DNA polymerase activity, DNA-dependent DNA polymerase activity, unwinding activity and, very importantly, RNase H activity that degrades RNA in RNA:DNA hybrids. Thus, there is no need to perform an additional time-consuming RNase H incubation step after reverse transcription, which shortens the reaction time. Single-stranded RNA as well as ssDNA are accepted as template and are reverse transcribed in the presence of a primer. The sequences of primers used were as follows: Forward'GGT CCT

ATG GAC TGA GTC CAGG, Reverse' TGA TTC CTC TGC ACA ACC TAA. PCR was performed using a PCR master mix (Sigma) and an annealing temperature of 55°C for 40 cycles. The PCR product was visualized by 1% agarose gel electrophoresis. The samples were run in triplicates for statistical analysis. An initial experiment was carried out to confirm the presence or absence of the GnRH receptor-1 in different tissues of the *Catla catla*, in absence of GnRH-1 injections. Subsequent experiments were carried out to detect the expression of the receptor following GnRH-1 administration and in normal saline injected animals.

Statistical Analysis

Comparison between groups were performed using one-way ANOVA with $p<0.05$ as the criterion for significance. All analysis was done using windows based SPSS statistical package (version 12.0, Chicago, IL).

Result

The results obtained are illustrated in Figures 1 and 2. Our gene expression analysis (PCR) in *Catla catla* various organs (Ovary, liver, brain and heart) revealed the existence of 5 hrs and 10 hrs treatment variations in the expression of *GnRH-1* in the control group (Figure 1). In both cases ovary, liver, brain and heart mRNA expression was lower at 10 hrs treatment and increased significantly at 5 hrs, in exhibiting the highest transcript levels at minimum hour's effect.

Determination of optimum doses of Gonopro-FH

In order to determine the relative GnRH receptor-I mRNA expression levels in different tissues as a result of Gonopro-FH injection and saline injected Catla fish were sacrificed at 5 hours and 10 hours. The 5-hour time point was selected on the basis of previous reports, where it has been shown that maximum efficiency of ovaprim-induced spawning occurs between 4-6 hours (Sridhar *et al.,* 1998). A varied degree of responses was observed in relation to the different doses of hormone. Female brooders showed a chasing behavior after 5-10 hrs. of injection of Gonopro-FH. None of the control fished spawned, however all the groups were injected hormone 0.45 ml/kg spawn successfully.

Effect of Gonopro-FH on egg production

Analysis of variance showed a significant effect ($P < 0.05$) of hormonal doses on egg output.

Discussion

In this work, we have determined the mRNA expression of *GnRH-1* receptors in the ovary, liver, brain and heart of the *Catla catla* by using mRNA expression. Our findings revealed the 5 hrs and 10 hrs treatment variations existence expression of *GnRH-1* receptors, which exhibit higher mRNA levels at 5 hrs. Although tempting, extrapolation of changes in transcript levels into similar changes in the biologically active peptide/protein levels requires further analysis.

Breeding behavior of fish is controlled by various environmental factors such as light temperature, pH, dissolved oxygen, meteorological condition (Bayarri *et al.* 2009). Ovaprim is a drug that is popularly used for induced breeding in fish (Peter *et al.,* 1988). Although it is successful in all major carps, the success rate has been rather low in catfish. In an attempt to investigate the rationale behind the low success rate in *Catla catla,* we studied the effect of Gonopro-FH on the distribution of GnRH receptors in the Catla fish, especially on its expression pattern in the ovary, in response to Gonopro-FH injection. Time-dependent expression profiling analysis revealed that Gonopro-FH injection decreased the expression of GnRH receptor-I mRNA in *Catla catla* ovary in a time-dependant manner. This observation may be attributed to a specific negative feedback mechanism imposed by the hormone-analog on the hypothalamo-pituitary-gonadal axis, which activates a signal that downregulates the expression of the GnRH receptors in the ovary.

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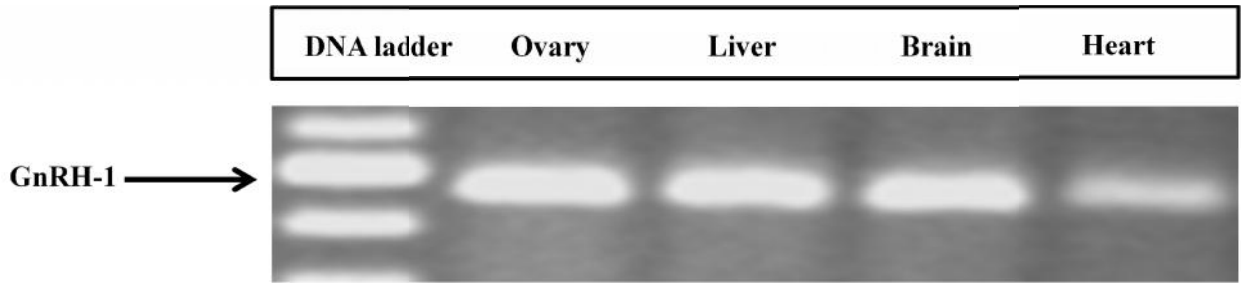

Figure 1. Relative *Catla catla* GnRH-1 receptor mRNA levels in different tissues

Expression levels were analyzed by RT-PCR using primers based on *Catla catla* GnRH-1 receptor. Maximum expression was observed in the ovary and minimum in the heart.

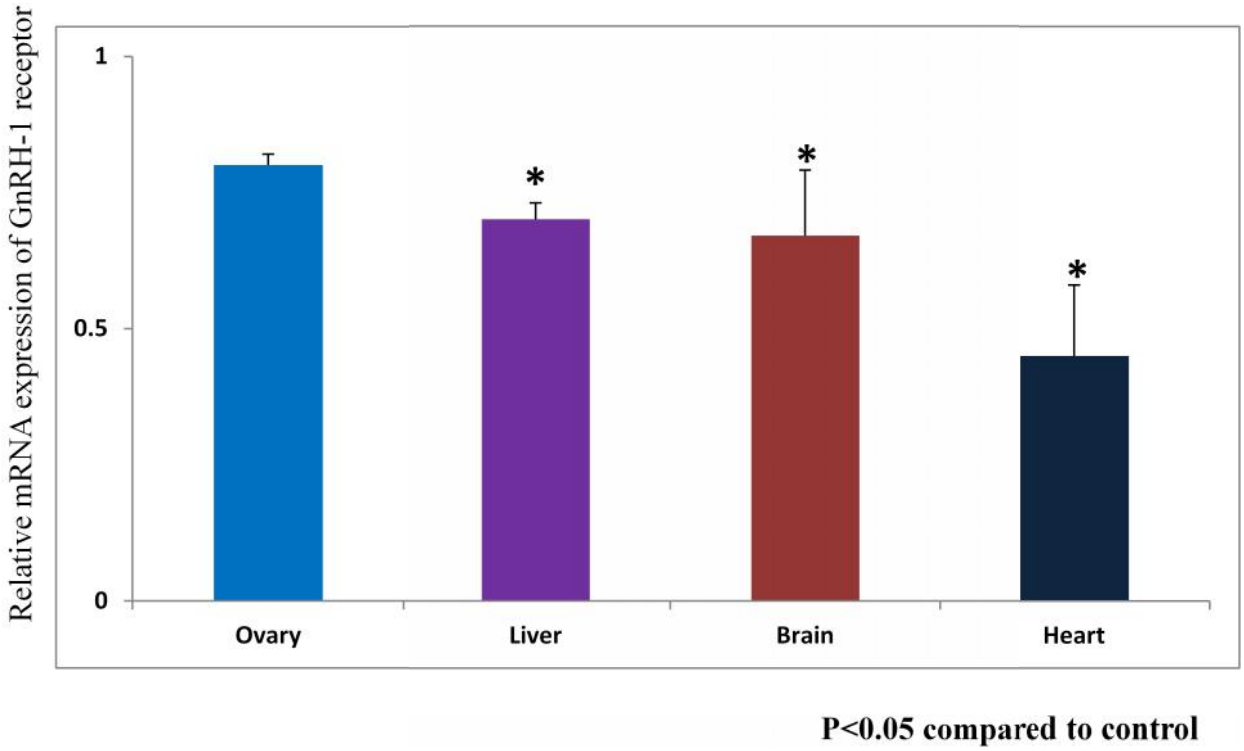

Figure 1 a. Graphical representation of the relative mRNA gene expression of GnRH-1 receptor in different tissues of *Catla catla using RT-PCR*

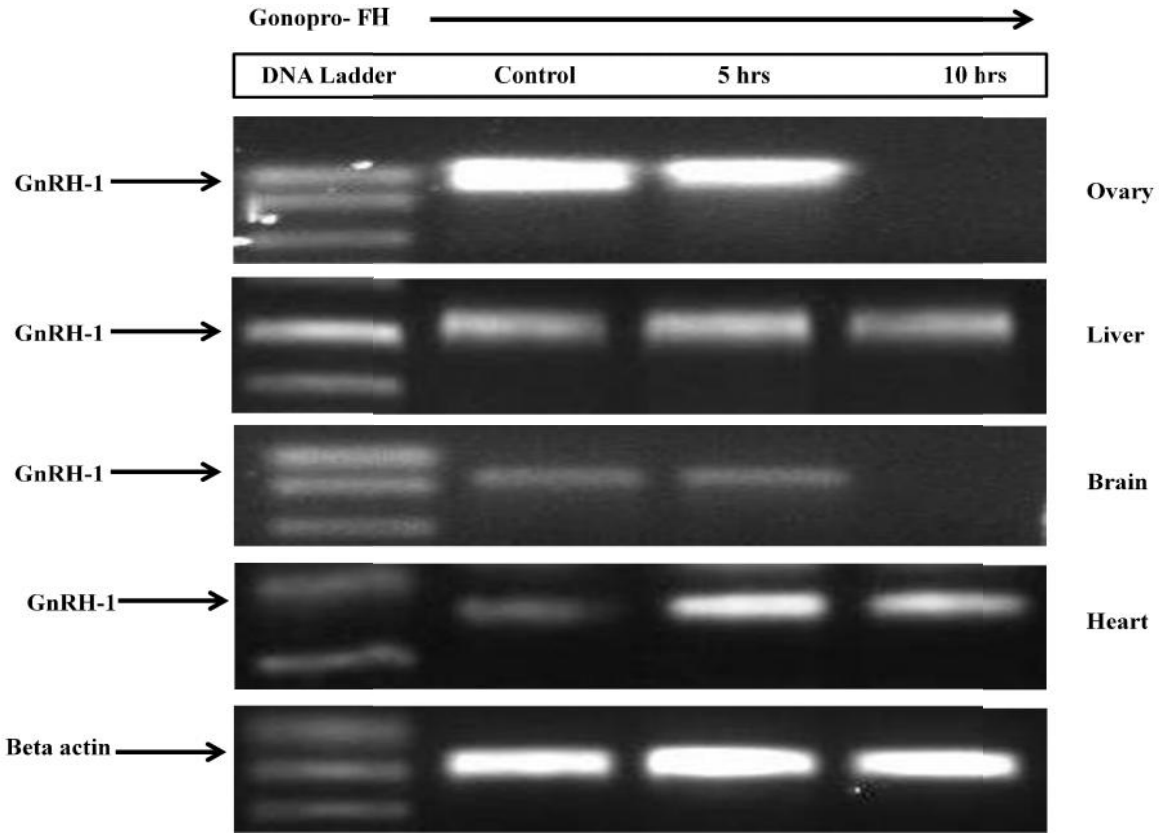

Figure 2. Effect of GnRH-1 receptor treatment on different tissues of the *Catla catla*

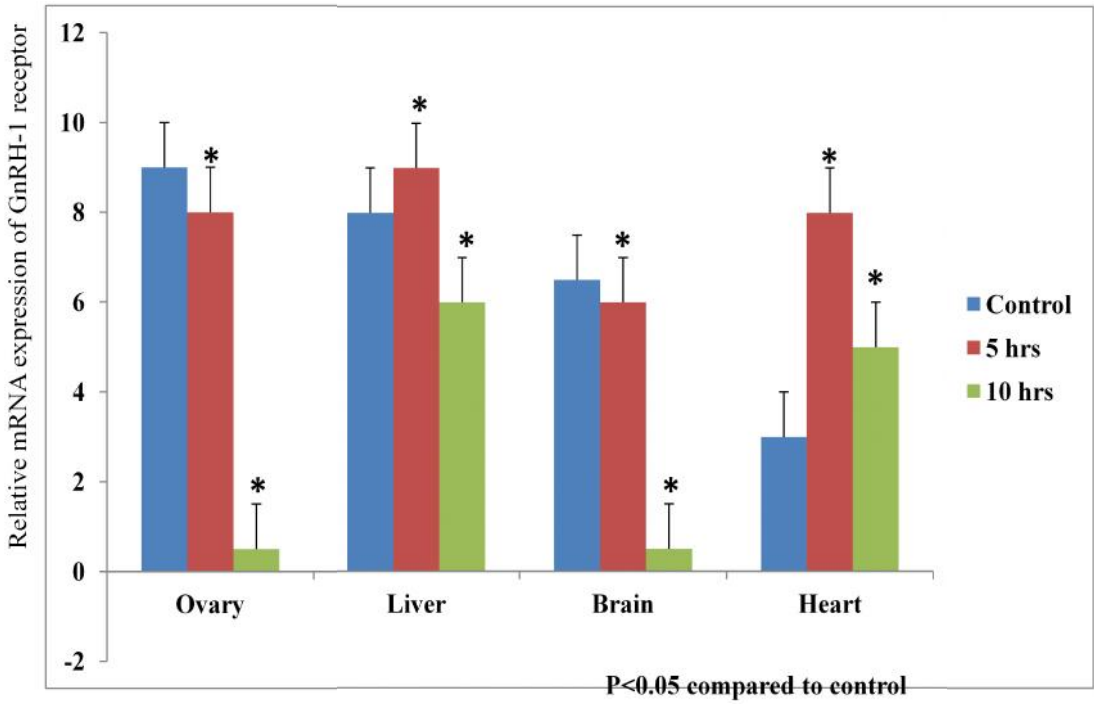

Figure 2 b. Graphical representation of the levels of GnRH-1receptor mRNA in different tissues of *Catla catla* after treatment with Gonopro-FH