



HOMOLOGY MODELLING AND DOCKING STUDIES ON REPRODUCTIVE HORMONES AND RECEPTORS OF *CLARIAS BATRACHUS* (LINNAEUS, 1758)

Dissertation submitted in
partial fulfillment of the requirements
for the degree of

M. F. Sc. (Fish Biotechnology)

By

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JUNE 2015



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Dated: 30/6/2015

CERTIFICATE

Certified that the dissertation entitled "Homology Modeling and Docking Studies on Reproductive Hormones and Receptors of *Clarias batrachus* (Linnaeus, 1758)" is a record of independent bonafide research work carried out by **Mr. Gulshan Kumar** during the period of study from August 2014 to June 2015 under our supervision and guidance for the degree of **Master of Fisheries Science (Fish Biotechnology)** and that the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title.

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I hereby declare that the dissertation entitled “**HOMOLOGY MODELLING AND DOCKING STUDIES ON REPRODUCTIVE HORMONES AND RECEPTORS OF *CLARIAS BATRACHUS* (LINNAEUS, 1758)**” is an authentic record of the work done by me and that no part thereof has been presented for the award of any degree, diploma, associateship, fellowship or any other similar title.

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ACKNOWLEDGEMENTS

I express my heartfelt gratitude and thanks to my Advisor, seniors, friends and staffs, who extended their knowledge to me along my dissertation work. I am thankful to my Institute, CIFE for providing everything which I required to build my academic career.

*I would like to convey my sincere respect, deepest honor, heartfelt gratitude and love to my supervisor **Dr. Aparna Chaudhari**, who helped me throughout my dissertation work. Without the help and proper guidance of my supervisor, the dissertation work would not have been completed successfully. It was possible to bring out this dissertation manuscript successfully because of his constant support, suggestions, guidance, encouragement, care and affection.*

*I also express my gratitude and thanks to my advisory committee members **Dr. Gireesh Babu P.** and **Dr. Dinesh Kumar**, for their uninterrupted encouragement, useful suggestions and kind help during the entire period of my research work.*

*It is my honor and great privilege to express my deep sense of gratitude to **Dr. W.S. Lakra**, Director, **Dr. A.K. Pal**, Joint Director and **Dr. Gopal Krishna**, HOD, CIFE, for providing the necessary facilities needed for conducting the study.*

*I am bound and privileged to mention the names of **Sujit Sir**, **Deepak Sir**, and **Mukesh Sir** for their unconditional and untiring support, love, care and guidance during the entire research work. To them I offer my heartiest gratitude and thankfulness.*

I shall ever remain indebted to my classmates. I have no words to express my sincere thanks for their immense help at each and every step of my dissertation work. The affection, assistance and encouragement

*provided by my classmates **Sudhanshu, Nilav, Nishant, Gunesha, Priyanka, Manu, Prawesh, Suhana, Mrinmoyee, Amol ,Jitendre, Gopal and Anupam** will always be remembered. I am also thankful to my juniors for their help and love.*

*I wish to mention the names of all the staff members of the FGB Division, **Sanjeev Ji, Reshma Ma'am, Ishita Ma'am, Yogita Ma'am, Biswas bhaiya, Patil, Jena Bhai**, who had all bestowed their valuable time in cooperation, support and helped me throughout my research period.*

I sincerely acknowledge the ICAR and CIFE for providing me fellowship without which I would not have been in a position to complete this work. I offer my indebtedness to the library staff for their timely help in providing books and journals necessary for my research work.

Last but not the least; I would like to thank The Almighty Lord, "The Supreme Scientist" of all science who had helped me during my entire stay at CIFE.

(GULSHAN KUMAR)

*30th June 2015
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सार

भारतीय मीठा पानी जलकृषि मुख्य रूप से कार्प पर निर्भर है और तीनों भारतीय मेजर कार्प कुल अंतःस्थलिय मछली उत्पादन में प्रमुख योगदान कर रहा हैं। हालांकि अच्छा स्वाद एवं उच्च पोषक महत्व के कारण कैटफिश का घरेलू मछली बाजार में कीमत ज्यादा है परन्तु इसके जलकृषि का व्यापक प्रसार नहीं हो पाया है। कैटफिश के बीज की अनुपलब्धता इसके जलकृषि के रास्ते में बड़ी बाधा है। शक्तिशाली संस्लेशी हॉर्मोन प्रेरित प्रजनन के लिए महत्वपूर्ण हैं और इसका उपयोग व्यापक रूप से कार्प बीज उत्पादन के लिए किया जाता है। हालांकि, नर *क्लैरिअस बेटराकस* को सालमोन GnRH आधारित फार्मूलेशन इंजेक्ट करने के बाद भी मत्स्य शुक्र निर्मुक्त नहीं होता है इसी कारण से मत्स्य शुक्र संग्रह करने के लिए नर का बलिदान किया जाता है। यह आनुवंशिक चयन कार्यक्रम में बहुत बड़ी बाधा है क्योंकि केवल माता मछली ही जिन्दा बचती है। GnRH एक संरक्षित अणु है, लेकिन यह संभव है कि *सी बेटराकस* GnRH और अन्य प्रासंगिक एंजाइमों/हॉर्मोन की बंधन बंधुता हेट्रोलॉगस ग्राही के लिए अलग हो। वर्तमान शोध में अन्य कैटफिश से सूचित इन दृश्यों (सीक्वेंस) की आम सहमति क्षेत्रों (कॉन्सेंसस रीजन) से डिजाइन किए गए पतित (डीजेनेरेट) पीसीआर प्राइमरों का उपयोग करके *सी बेटराकस* के गोनाडोट्रोपिन हॉर्मोन अल्फा सबयूनिट (GtHa, 201 बीपी), फॉलिकल स्टिम्युलेटिंग हॉर्मोन β सबयूनिट (FSH β ; 213 bp), गोनाडोट्रोपिन रीलीजिंग हॉर्मोन ग्राही (GnRH-R, 342 bp) एवं luteinizing हॉर्मोन ग्राही (LH-R; 1064) का आंशिक दृश्य उत्पन्न किया गया। *सी बेटराकस* की FSH β और LH β हॉर्मोन की *सी गेरीपीनस* के इसी हॉर्मोन के ग्राही के साथ बंधन क्षमता निर्धारित करने के लिए अनुरूपता मॉडलिंग और डॉकिंग किया गया और पाया गया की *सी बेटराकस* LH β होमोलॉगस जोड़ी की तुलना में हेटरोलोगस LH ग्राही को अधिक कुशलता से बांधता है। अन्य सभी बंधन समान थे। जब सालमोन, चिकन और कैटफिश GnRH का परिक्षण सालमोन ग्राही और कैटफिश (*सी gariepinus*) GnRH-R1 और R2 के साथ किया गया तो एक महत्वपूर्ण अवलोकन पाया गया। *सी बेटराकस* GnRH का कैटफिश GnRH-R2 के साथ बहुत मजबूत अन्योन्यक्रिया है तथा सालमोन ग्राही के साथ केवल साधारण बंधन पाया गया। इससे भी ज्यादा महत्वपूर्ण की सालमोन GnRH का कैटफिश GnRH ग्राही के साथ साधारण अन्योन्यक्रिया था।

Abstract

Indian freshwater aquaculture is mainly carp based and the three Indian major carps are major contributors to the total inland fish production. Though catfishes fetch a good price in the domestic fish market due to good taste and higher nutritional value, their aquaculture is not wide spread. Unavailability of seed for these catfishes is the major hurdle faced by fish farmers. Potent synthetic hormones are the key to induced breeding and they are widely used for carp seed production. However, the *Clarias batrachus* male does not release milt on being administered salmon GnRH based formulations and has to be sacrificed for milt collection. This also constraints genetic selection of the species for improved traits as only one parent survives. Although GnRH is a conserved molecule, it is possible that the *C. batrachus* GnRH and other relevant enzymes/hormones have significantly different binding affinities to heterologous receptors. In the present work, partial sequences of *C. batrachus* gonadotropin hormone alpha subunit (GtH α ; 201 bp), follicle stimulating hormone β subunit (FSH β ; 213 bp), gonadotropin releasing hormone receptor (GnRH-R, 342 bp) and luteinizing hormone receptor (LH-R; 1064) were generated by using degenerate PCR primers designed from consensus regions of these sequences reported from other catfishes. To determine the 3' & 5' transcript termini, rapid amplification of cloned ends (RACE) procedure was adopted. Only GtH α and FSH β sequences could be extended by 274 and 456 bp, respectively, on the 3' ends. 5' ends could not be obtained for any partial sequence. Homology modelling and docking studies were done to determine binding efficiencies of *C. batrachus* FSH β and LH β with corresponding receptors from *C. gariepinus* and it was found that *C. batrachus* LH β binds more efficiently to the heterologous LH-R compared to the homologous pair. All other bindings were similar. A significant observation was made when binding of salmon, chicken and catfish GnRH was tested with salmon receptor and catfish (*C. gariepinus*) GnRH-R1 and R2. The *C. batrachus* GnRH showed much stronger interaction with catfish GnRH-R2 and only moderate binding to salmon receptor. More importantly, the interaction of salmon GnRH with catfish GnRH receptors was only moderate.

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1. INTRODUCTION

Aquaculture is one of the fastest growing food industries and aquatic animals are an important source of protein rich food throughout the globe (Ayyappan *et al.*, 2006). To meet the rising demand, aquaculture production should increase rapidly through diversification of cultured species, genetic improvement and intensification of culture practices. India ranks second in total global fish production with a total of 9.58 MT of fish production, in which inland sector contributes around 6.14 MT (Department of Animal Husbandry Dairying and Fisheries, 2015). Though India has a rich biodiversity of aquatic animals, Indian aquaculture is primarily carp dependent, although shrimps are also being cultured in freshwater currently. Lack of quality seed and feed, absence of genetically improved strains and non-standardized aquaculture practices are the major lacunae that are preventing widespread cultivation of alternative species. *Clarias batrachus* commonly referred to as the Indian magur, is a native catfish of India, whose culture is restricted on account these reasons. Although it enjoys a good demand due to its good taste and nutritional value and fetches a good price in many local markets in India, its aquaculture production is very low.

Owing to its air breathing nature, *C. batrachus* generally lives in stagnant water bodies and even in oxygen depleted conditions. It matures after 1 year and weighs ~ 150 gm. There is sexual dimorphism where males and females are distinguished externally through the shape of genital papilla. It breeds only once in a year from June to August and has very low fecundity in the range of 15000 - 20000. The female lays eggs in pits made along the margins of water bodies. Parental care is seen and male guards the eggs and young ones for a few days. Both males and females mature in captivity and on induction by gonadotropin releasing hormone (GnRH) females release eggs on stripping. Males, however, do not readily ooze milt on stripping and need to be either sacrificed or surgically operated to obtain testis that are macerated to obtain sperms (Thomas, 2003). This problem obstructs the implementation of suitable genetic selection program.

Successful breeding and quality seed production technology go a long way towards developing a sustainable production system for any commercially important fish. Regulation of several external and internal factors affecting reproductive physiology, like photoperiod, water flow, temperature, hormones, etc, are essential for breeding fishes in captivity. Use of synthetic inducers is presently a common aquaculture practice that is indispensable in most cases. However, all fishes do not respond in a similar manner because of their specific endocrine regulatory mechanisms. More potent biomolecules are required for species like mangur and this can be done by implementing the concepts of structural biology to identify and design new molecules targeted to specific needs.

Full length coding sequences of *C. batrachus* GnRH, luteinizing hormone β subunit (LH β), cytochrome 450 aromatase and vasa genes known to play major roles in reproductive physiology, have been elucidated in our lab recently. GnRH and LH β interact with receptors that have not yet been sequenced. In addition, *C. batrachus* follicle stimulating hormone β subunit (FSH β) and the gonadotropin α subunit (GtH α) have also not been sequenced. In this context, the present study is planned with the following objectives.

Objectives

- ◆ Amplification and sequencing gonadotropin releasing hormone receptor and gonadotropin hormone receptor genes of *Clarias batrachus*.
- ◆ Homology modelling and docking studies on reproductive hormones and receptors of *Clarias batrachus*.

2. REVIEW OF LITERATURE

2.1. *Clarias batrachus* and its Importance in Aquaculture

Clarias batrachus commonly called magur is native to SE Asia and has been introduced into many other places for fish farming (Lever, 1996). It is an opportunistic feeder, can go for months without food and has been described as a benthic, nocturnal, tactile omnivore that consumes detritus and opportunistically forages on large aquatic insects, tadpoles, and fish. It naturally occurs in lakes, water courses, wetlands, medium to large rivers, swamps, ponds, ditches, flooded fields and rice paddies. It is a tropical species with a moderate tolerance to colder waters and reported to have a low lethal temperature of 9.8°C. During cold dry months, magur burrows into the sides of ponds and streams where they remain dormant until the spring rains begin (Masterson, 2007).

Catfish meat, especially *C. batrachus* is prescribed prophylactically to anaemic and malnourished individuals as well as for convalescent patients due to its nutritional superiority (Debnath, 2011). It is considered a medicinal fish and traditionally advised for pregnant and lactating mothers, the elderly and children. A common perception of easily digestible high grade protein, high iron and beneficial lipid content are most likely the reasons behind this. A year-long study on the blood plasma lipid of *C. batrachus* in a population (Debnath, 2011) revealed that the HDL content ranges from 150 mg/dl – 180 mg/dl which is more than 60% of total cholesterol. The comparatively simple culture conditions characterized by efficient food conversion (Ali & Jauncey, 2005) and excellent nutritional profile (Rui *et al.*, 2007) make magur very suitable for commercial intensive culture. Successful aquaculture of this species may bring about socioeconomic sustainability of the rural people. However, availability of seed is a major issue as natural collections are fast diminishing and induced breeding protocols for *C. batrachus* have not achieved the simplicity of carp breeding. The total inland fish production of 2013 – 14 is 6.14 million tons (DAHDF, GoI, 2015) of which the majority share, > 80% is of carps.

Specific catch data of *C. batrachus* is not documented

Unlike other species where a mature male releases copious quantities of milt after injecting with gonadal steroid, the multi-lobed catfish testis does not produce spontaneous release of milt even by stripping. The use of synthetic inducing agents for successful ovulation followed by stripping of the female catfish is a common practice and has been studied at several occasions (De Leeuw *et al.*, 1985, Manickam and Joy, 1989, Tan-Fermin *et al.*, 1997). However, results of induced spawning remain highly unpredictable in male catfish. Therefore, the males have to be sacrificed to obtain milt and this act as a major bottleneck for breeding and ultimately hamper production of seed for aquaculture.

2.2. Fish Gonadotropins

Levavi-Sivan *et al.* (2010) have comprehensively reviewed fish gonadotropins (GtH) and their receptors. According to them, LHb and FSHb cells are found in proximal pars distalis of the pituitary. The gonadotropic activity of fish pituitary extracts was confirmed in early 1960s by its capacity to maintain gonadal functions in mammals. Gonadotropin hormone was partially purified from carp and salmonid pituitaries in the late 1960s and early 1970s (Burzawa-Gerard, 1971; Donaldson *et al.*, 1972) by purifying the glycoprotein fraction on ion exchange chromatography and gel filtration columns. The first gonadotropin purified was similar to GtH2 and was later renamed as luteinizing hormone (LH). The presence of two gonadotropins was first demonstrated in chum salmon (*Oncorhynchus kata*) pituitary (Suzuki *et al.*, 1988) and two forms could later be purified from several fishes by affinity chromatography of the glycoprotein fraction. They were first named GtH1 and GtH2, but later renamed as follicle stimulating hormone (FSH) and LH. Both the hormones were confirmed to be heterodimers with molecular weights ranging from 30 to 50 kDa. Binding studies with specific antibodies revealed the presence of one α -subunit (glycoprotein α ; GP α) that is common to both FSH and LH hormones. The other subunit that is specific to each hormone was named FSH β and LH β (Pierce and Parsons, 1981). The common α subunit binds to specific β

subunit non-covalently, but with high specificity and avidity. The α -subunit gives structural support, while β -subunit determines the biological activity and specificity of each hormone. Each subunit is encoded by different genes. All the three subunits viz. α , FSH β and LH β have come from three different evolutionary lineages, but because of their sequence similarity, and their significant structural relationship it has been suggested that there is a common origin for all three subunits (Querat *et al.*, 2000). Further evolutionary analysis has revealed that both α - and β -subunits evolved from a single ancestor through gene duplication more than 900 million years ago (Li & Ford, 1998). Due to the presence of a single gonadotropin β -subunit-like cDNA in lamprey, it has been suggested that an ancestral β subunit has given rise to LH β and FSH β during early evolution of the gnathostomes (Sower *et al.*, 2009). According to the results obtained from hormonal assays and expression profiling, it has been revealed that FSH β has its importance in early stages of gametogenesis, while LH β plays a role during ovulation and spermiation.

2.3. Fish Gonadotropin Receptors

The results of binding studies conducted in the early 1970s, using mammalian gonadotropin (human chorionic gonadotropin, hCG) and purified hypophysial glycoproteins, revealed the presence of specific membrane-bound receptors (GtHRs) in fish gonads (Breton *et al.*, 1971). Binding of fish LH to its high-affinity receptors was demonstrated first in the salmonid ovary (Breton *et al.*, 1986; Kanamori *et al.*, 1987; Salmon *et al.*, 1983) and in the trout testis (Le Gac *et al.*, 1988). Coding sequences corresponding to two different GtHRs were reported from several fish species, including salmon (Maugars & Schmitz, 2006; Oba *et al.*, 1999), catfish (Bogerd *et al.*, 2001; Kumar & Trant, 2001; Vischer & Bogerd, 2003), zebrafish (Kwok *et al.*, 2005; So *et al.*, 2005), sea bass (Rocha *et al.*, 2007), eel (Jeng *et al.*, 2007), and trout (Sambroni *et al.*, 2007). Data obtained from whole genome sequencing of different fish species belonging to different taxa confirmed the presence of two single copy genes for each gonadotropin receptor, FSHR and LHR. The two receptors are G-protein-coupled receptors (GPCR) belonging to the

family of rhodopsin-like receptors (family A) (Gether, 2000). Together with thyroid hormone receptor (TSHR), they constitute the subfamily of glycoprotein hormone receptors (GpHR). These receptors consist of three structural domains, including a large extracellular domain, a transmembrane domain with seven helices and a carboxy-terminal intracellular domain (Vassart *et al.*, 2004). Fish GtHRs also have the same general structure, but their extracellular domains differ significantly from mammalian FSHRs. The larger extracellular domain of these GpHRs contributes more than half of the length of the protein, and is responsible for the hormonal recognition and binding. The most conserved part in these receptors is the transmembrane domain with seven α -helices. Each α -helix contains 22–28 hydrophobic amino acids, which extend across the lipid bilayer and the helices are connected by three intracellular and three extracellular loops. The transmembrane domain is important for receptor activation and signal transduction (Ulloa-Aguirre *et al.*, 2007; Vassart *et al.*, 2004). Intracellular carboxy terminal domain has its function in G protein coupling, directing fate of internalized receptor and binding of different intracellular signaling molecules (Ulloa-Aguirre *et al.*, 2007). There is significant sequence variability in the intracellular domain among fish GtHRs. The phylogenetic tree when constructed by taking amino acid sequences from vertebrate GtHRs and TSHRs revealed the presence of two main clades corresponding to TSHRs and GtHRs which suggests that these receptors have evolved from the duplication of a common ancient gene. A more detailed phylogenetic analysis revealed that FSHR and LHR segregate together compared to the TSHRs to form two subclades. This suggests that both GtHRs emerged subsequently from the duplication of a second common ancestral gene (Reviewed by Levavi-Sivan *et al.* 2010). In mammals FSHR and LHR generally bind specifically to their corresponding ligands and show little cross reactivity, but in fishes the situation is different because of promiscuous hormone–receptor interactions. This promiscuous binding was first demonstrated for coho salmon where receptor-ligand binding studies using radiolabelled hormones have shown that the FSHR molecules localized on both the theca and granulosa cells do not differentiate between FSH and LH binding, while the LHR localized on

granulosa cells, bind only LH (Miwa *et al.*, 1994; Yan *et al.*, 1992).

2.4. Gonadotropin Releasing Hormones and Receptors

Lethimonier *et al.* (2004) have comprehensively reviewed gonadotropin releasing hormone (GnRH) and its receptors in fishes. GnRH has its importance in fish farming because it is used as an inducing agent during induced fish breeding. Due to presence of multiple isoforms of both GnRH and GnRHRs, teleost fishes are considered good models for understanding the evolutionary mechanisms of both GnRH and its receptor genes in vertebrates. Based on the evidences that a hypothalamic factor stimulates the release of pituitary gonadotropic hormone in carp (Breton *et al.*, 1971), the first GnRH was characterized from salmon (Sherwood *et al.*, 1983). Studies on binding properties of GnRH have been carried out on gold fish by Habibi *et al.* (1987). First fish GnRH receptor (GnRH-R) was cloned from catfish by Tensen *et al.* (1997). After these pioneering studies, a large number of GnRH variants have been reported from vertebrates. Teleost fishes have the maximum number of GnRH variants among vertebrates. First identified fish GnRH form was salmon GnRH (sGnRH; Sherwood *et al.*, 1983), after which 7 other GnRH forms were identified in teleosts by using the techniques of high performance liquid chromatography (HPLC) and sequencing. Fishes contain mammalian GnRH, identified in fish for the first time in eel (King *et al.*, 1992), chicken GnRH II, identified in goldfish (Yu *et al.*, 1988) and 6 additional fish specific forms that include catfish GnRH (cfGnRH). Similar to other vertebrate classes, the teleost fishes also contain at least two GnRH variants in their brain. In some cases, even more than two variants have been reported from a single fish. As now reported for all vertebrate classes, the brain of teleosts contains at least two GnRH variants, but there is a growing number of species in which three GnRH variants have also been reported as in seabream (*Sparus ourata*; Gothilf *et al.*, 1996). Catfish GnRH is found in olfactory bulbs, telencephalon and preoptic area and chicken GnRH (cGnRH II) has been detected in midbrain tegmentum in *Clarias gariepinus* (Zandbergen *et al.*, 1995). Nascent GnRHs contain four different regions which include a signal peptide

(around 20–25 residues), the biologically active GnRH decapeptide sequence, a processing tripeptide (Gly–Lys–Arg) and the GnRH associated peptide (GAP; ~4050 residues). Except the biologically active decapeptide rest of the sequences are cleaved off to form mature biologically active hormone.

The first full length cDNA encoding GnRH receptor (GnRH-R) was reported by Tensen *et al.* (1997). Since then, cDNA encoding GnRH-R have been characterized in various teleost fishes including goldfish, rainbow trout, medaka, striped bass, amberjack, African cichlid, European sea bass, and Japanese eel (reviewed by Lethimonier *et al.*, 2004). GnRH-R, belongs to the beta subgroup of rhodopsin subfamily of G protein coupled receptor family. Being a part of GPCR, GnRH-R also shows similar topology containing three main functional domains that include one N-terminal extracellular domain (30–40 aa), a large transmembrane domain (280–290 aa), and a short C-terminal cytoplasmic domain (30–50 aa). The transmembrane domain consists of seven highly conserved transmembrane alpha helices that are required for anchoring the receptor to the cell membrane. The segments between these transmembrane helices form extracellular and intracellular loops which have an important role in ligand binding and signal transduction. Phylogenetic analysis of available GnRH-R sequences from various teleost fishes has indicated the presence of two main types termed Type I and Type II (reviewed by Lethimonier *et al.*, 2004). The two types of GnRH-R have divergent motifs, notably in the transmembrane (TM) domain (reviewed by Lethimonier *et al.*, 2004). All Type I receptors have a C/GAFVT motif in TM helix-3 and DLE-GKVSHSLTH like sequence at the beginning of TM helix-7, whereas Type II ones contain a SAFIL motif in TM helix-3 and a VTPEY motif in TM helix-7 (reviewed by Lethimonier *et al.*, 2004). It is clear from reports that teleost fishes have two different GnRH-Rs i.e. GnRH-R I and GnRH-R II with subtypes reviewed by (Lethimonier *et al.*, 2004). Signal transduction from GnRH-R involves G-protein and membrane bound phospholipases (reviewed by Lethimonier *et al.*, 2004) that results in an increase in cytosolic diacylglycerol (DAG) and inositol 1,4,5- triphosphate (IP3), leading to efflux of calcium from intracellular stores. GnRH-R may also involve cAMP and

arachidonic acid. From the binding studies carried out in different fish species, it has been demonstrated that different GnRH-Rs bind with different GnRH variant with varying affinities (Robison *et al.*, 2001; Bogerd *et al.*, 2001). In studies carried out in transfected cells, different GnRH peptides have resulted in activation of both the receptors. There is clear preference in terms of sensitivity for cGnRH-II, followed by sGnRH and a third endogenous GnRH form (species specific) for both the receptors. The expression of GnRH-R genes is wide, but mainly restricted to reproductive tissues. In a single fish species there may be differences in tissue and/or cellular distribution for each GnRH-R type and subtype. In catfish, two GnRH-R variants named GnRH-R1 and GnRH-R2, both belonging to type II category, have been reported. Both receptors bind more strongly to cGnRH II than cfGnRH. GnRH-R1 is expressed in pituitary > brain > testis, while GnRH-R2 is expressed in brain > ovary > heart > testis > pituitary.

2.5. Gene Characterization

A number of strategies can be used for sequencing unknown mRNA molecules (Brown, 2010). However, PCR based approaches are easiest to adopt and execute in the present scenario where a large number of sequences from related organisms are available in the public database. Degenerate PCR primers can be designed in the consensus regions of the sequences reported from closely related organisms, and a fragment of the gene of interest is amplified using cDNA prepared from the appropriate tissue. The fragment is used to generate a partial sequence against which gene specific primer can be designed for 5' and 3' rapid amplification of cDNA ends (RACE). This technique is used to identify the 5' and 3' termini of partial RNA sequences. Schaefer (1995) has reviewed several variations of RACE amplification procedure. These include anchored PCR, one sided PCR, ligation anchored PCR (LA-PCR), single-stranded ligation to single stranded cDNA ends (SLIC), RNA ligase-mediated RACE (RLM-RACE) and reverse ligation-mediated PCR (RL-PCR) (reviewed by Schaefer 1995).

RACE procedure for cloning unknown 5' and 3' cDNA ends was described initially by

Frohman *et al.* (1988) and at the same time Loh *et al.* (1989) described the anchored PCR (A-PCR) procedure for cloning the 5'-ends of T cell receptor genes which have a constant 3'-region but variable 5'-sequences. Basic procedure of both groups was nearly identical. In both the procedures first strand cDNA had been synthesized from RNA by using either oligo(dT) primer or a primer complementary to known regions of the target mRNA. The 3' end of first strand cDNA was then modified by homopolymeric tailing using terminal deoxynucleotidyl transferase (TdT). Second strand cDNA was then synthesized by using a primer having an RE site in its 5'-region. This double stranded cDNA was then amplified by PCR using cloning adaptor (anchor) as 5'-end primer and a gene specific primer complementary to the known internal cDNA sequence. Amplification of 3'-terminus termed as 3'-RACE, involves first strand cDNA synthesis by oligo(dT) primer, followed by amplification using a gene-specific primer and oligonucleotide primer complementary to oligo(dT) or oligo(dt) adaptor primer.

Vischer *et al.* (2003) have cloned the complete cDNA of FSH β subunit in African catfish and also observed spatiotemporal expression profile of this hormone. The authors used a cDNA library prepared from mRNA of a mature male pituitary and screened it using colony PCR with degenerate primers. The missing 5' end was identified by 5' RACE using SMART RACE kit (Clontech, USA). The authors also used a genomic DNA library constructed from liver tissue to sequence the complete gene. Degenerate primers based on conserved sequences of goldfish (*Carasius auratus*) and Channel catfish (*Ictalurus punctatus*) were used. PCR products were compared and exons and introns were identified. This revealed the presence of three exons, interrupted by two introns in *cfFSH β* gene.

Liu *et al.* (2001) sequenced of FSH and LH genes of channel catfish (*Ictalurus punctatus*) and also did expression profiling of these genes during ovulation. A pituitary cDNA library prepared in Unizap λ vector was screened for clones carrying desired sequences. The fixed orientation of cDNAs in the plasmid vector allowed the use of vector-anchored PCR for determining 5' and 3' termini.

The transcripts for the channel catfish FSH and LH, encode proteins of 132 and 140 amino acids, respectively. The sequences show high homology sequences from other teleosts.

Cloning and functional characterization of LH-R cDNA from African catfish was done by Vischer *et al.* (2002). African catfish were bred and raised in the laboratory and catfish pituitary extract was used to induce ovulation. Genomic DNA was obtained from sperms of 10 to 12 month old catfish. This DNA was amplified using degenerate primers designed based on conserved amino acid sequences. One 0.3 kb fragment was generated, subcloned, and sequenced. Based on the DNA sequence obtained, two specific primers were designed to screen for cfLH-R in a cDNA library of catfish testis prepared in ZAP Express vector (Stratagene, USA). However, this method failed to yield the 5' end sequence and the authors used SMART RACE cDNA amplification kit (Clontech, USA) to obtain the complete sequence of 2447-base pair consisting of an open-reading frame of 2133 nucleotides, flanked by a leader sequence of 188 nucleotides and a trailer sequence of 126 nucleotides.

Kwok *et al.* (2005) have worked on characterization of genes encoding FSH-R and LH-R in zebrafish and have also collected evidences for their distinct function in follicle development. Using cDNA prepared from ovary of mature fishes as template the authors could amplify a 380 bp product with degenerate primers. The full length cds generated by SMART RACE (Clontech, USA) was 2965 bp in long having a 2127-bp ORF that encodes a protein of 708 residues. As LH-R was a transmembrane receptor, there was also a typical hydrophobic signal peptide of 21 amino acids at the N-terminal. The deduced amino acid sequence of zebrafish LH-R shared the highest homology (73%) with that of African catfish. FSH-R responded only to FSH but LH-R responded to both LH and FSH.

Maugars *et al.* (2006) worked on molecular cloning and characterization of FSH and LH receptors in Atlantic salmon. The design of primer sets for FSHR and LHR was based on salmonid FSHR and LHR sequences

available in the GenBank database and full length sequences were recovered by RACE. The authors could generate 2722 bp of LH-R cDNA that spans the complete ORF. The predicted protein sequence comprises a mature protein of 701 residues and a signal peptide of 27 amino acids at the N-terminal end.

Illing *et al.* (1999) characterized two GnRH-R genes from goldfish (*Carassius auratus*) and also showed their distinct ligand selectivity and differential distribution. The authors report the presence of two different GnRH receptors in the goldfish pituitary, because of their differences in stimulating GtH-II and growth hormone release by different GnRH analogues. Two full-length putative goldfish GnRH-R cDNAs, GfA and GfB, were identified in this study. Both showed the same order of potencies for natural GnRHs with affinities decreasing in the order cGnRH-II > sGnRH > mammalian GnRH > sea bream GnRH. However, GfA showed a greater preference for cGnRH-II and a lesser preference for the other natural GnRHs than did GfB. Both receptors are expressed in the pituitary, brain, ovary and liver. Sequence identity between GfA and GfB amino acids was 71%. There was 82% and 71% sequence identity with catfish GnRH receptor for GfB and GfA amino acids respectively.

Okubo *et al.* (2000) completed molecular cloning of the GnRH-R gene of Japanese eel (*Anguilla japonica*) and explored its tissue specific expression. Following the same approach, the authors first amplified a partial sequence of 570 bp in the TM domain using cDNA from the pituitary of an immature male eel, and then used RACE to obtain the full length sequence of 3233 bp. This comprised of a 320 bp 5' UTR and 1140 bp complete ORF. The GnRH-R was also sequenced from the genome and found to consist of three exons and two introns. The polypeptide sequence of the Japanese eel GnRH-R has 73, 73, and 72% amino acid identity with that of *C. gariepinus* receptor, *C. auratus* GfA and *C. auratus* GfB, respectively. Tissue specific expression was detected in various parts of brain, pituitary, eye and testis.

2.6. Homology Modelling and Docking

Principles and methodologies involved in homology modelling have been comprehensively explained by Xiong (2006). Due to increase in knowledge of proteomics and growth in computational power, bioinformatic tools are becoming popular for three dimensional structure predictions and their functional analysis. There are three computational approaches for the prediction of three-dimensional structure and modelling of proteins. They are homology modelling, threading and *ab initio* prediction. The first two methods predict protein structures based on knowledge of existing protein structural information in databases. In homology modelling, an atomic model is built based on experimentally determined structure of a protein having similar amino acid sequence to the target. Threading involves structural prediction based on structural similarity with or without detectable sequence similarities. The *ab initio* approach does not involve structural or sequence similarity but it predicts structures based on physicochemical principles involved in protein folding.

Homology modelling involves following sequential steps to make a three dimensional model based on structures of homologous proteins.

2.6.1. Template selection

A template is selected by searching the Protein Data Bank (PDB) for homologous proteins with experimentally determined structures. The criterion for the selection of suitable template is at least 30% sequence identity with the query sequence. Structures with the highest percentage identity, highest resolution, and the most appropriate cofactors should be preferred as templates.

2.6.2. Realignment and backbone model building

To obtain optimal alignment for the template and target sequence, realignment has to be done using more refined algorithms. It is assumed that aligned regions of the target protein can assume a similar structure as the template proteins. So the coordinates of the corresponding residues of the template proteins can be copied onto the target protein. Coordinates of even side chain atoms are copied

along with the main chain atoms when two aligned residues are identical.

2.6.3. Loop modelling

Gaps created due to insertion and deletion in the aligned sequences cannot be directly modeled and create “holes” in the model. Closing the gaps the database searching method and the *ab initio* method.

2.6.4. Side chain modelling

Side chain modelling involves searching of every possible conformation at every torsion angle. From the search output, the side chain having lowest interaction energy with neighboring atoms should be selected. The concept of rotamers, which are favored side chain torsion angles extracted from known protein crystal structures are utilized generally to obtain the most favorable arrangement.

2.6.5. Energy minimization

Energy minimization procedure is performed to the complete raw homology model to remove structural irregularities such as unfavorable bond angles, bond lengths, or close atomic contacts. This process moves atoms of the model in such a way that overall conformation should attain lowest potential energy. The structural anomalies should be corrected without significantly altering the overall structure. Another way for structural refinement is molecular dynamic simulation. It involves searching for global energy minimum by moving atoms uphill as well as downhill in a rough energy landscape.

2.6.6. Model evaluation

This is the final stage of homology modelling. This involves verification of structural features like ϕ – ψ angles, bond lengths, close contacts based on pre-existing physicochemical principles. The errors in the model are detected by compiling statistical profiles of spatial features and interaction energy from experimentally determined structures.

2.6.7. Protein- protein docking

Planas-Iglesias *et al.* (2012) have comprehensively reviewed the tertiary and quaternary structure prediction of proteins. In this paper they described the principle of protein docking. Protein docking is a computational technique for determining the structures of protein-protein complexes. These techniques involve sampling the orientation of two unbound protein structures to make several predictions about their interaction, followed by a scoring step to rank the predictions based on a scoring function determined on the basis of observed principles of protein-protein interactions. First step is a rigid-docking in which search is performed by treating the two proteins as rigid bodies. One of the proteins, called the receptor, is kept fixed while the other protein, the ligand, is rotated and translated around the first. Next, further refinement of some structures takes place, allowing changes in conformation of the two unbound structures upon binding.

2.7. Case Studies in Homology Modelling and Docking

Hossain (2012) computationally analyzed fish antifreeze proteins and also characterized their physicochemical characteristics. In this study, a total of 15 antifreeze proteins of fishes were selected that represent distinct physicochemical and structural features. Sequences were retrieved from Swiss-Prot, a public domain protein database by random selection. The physicochemical properties like theoretical isoelectric point (pI), molecular weight, total number of positive and negative residues, extinction coefficient (Gill & Von Hippel, 1989), half life (Tobias *et al.*, 1991), instability index (Guruprasad *et al.*, 1990), aliphatic index (Atsushi.,1980) and grand average hydropathy (GRAVY; Kyte & Doolittle, 1982) were computed using the Expasy's prot-Param prediction server. The amino acid compositions of all proteins were also determined with their physicochemical properties. The transmembrane domains were identified by SOSUI server and were visualized and analyzed using helical wheel plots. SOPMA was employed for calculating the secondary structural features. The presence of disulphide bonds and their bonding pattern were predicted by CYS_REC and What if server. Homology modelling for the

prediction of three dimensional structures was done by three homology modelling servers Geno3D, Swiss-model and CPH-models. The model was evaluated by Rampage, ProQ and ProSA. Structural validation was performed by online PROCHECK and What IF. The results of primary structural analysis suggested that requires loop modelling. There are two main techniques used for loop modelling viz. proteins from fishes were mostly hydrophobic due to presence of high non-polar residues. Most of the fish antifreeze proteins had basic character with an average pI 6.73. The instability indexes of antifreeze proteins ranged from 13.03 to 41.21 indicating their stability. Aliphatic index, which is a measure of thermal stability, indicated that thermal stability ranged from very high to very low. Analysis of Grand Average Hydropathy (GRAVY) revealed that all fish antifreeze proteins analyzed were hydrophobic. Among the secondary structure features, random coil dominated followed by alpha helix, extended strands and beta turns. A comparison of the results obtained from three above mentioned homology modelling servers, shows that the models generated by Swiss model were more acceptable in comparison with others based on Ramchandran plot analysis.

Santiago and Alavandi (2012) performed molecular modelling and docking on shrimp vitellogenin receptor and ligands including methyl farnesoate, farnesoic acid and 20-hydroxy ecdysone to analyze target mediated delivery system for *Monodon baculovirus*. It was demonstrated earlier that hepatopancreas is the site of synthesis of vitellogenin. The target receptor sequence was retrieved from NCBI and was first split into 4 chains and each of four chains one at a time was used for homology modelling using T-08 SAM SERVER. The quality of model was assessed using PROCHECK and 3D Verify. The 3D structure was visualized by RASMOL. Ligand molecule was obtained from drug bank. The 3D structure and energy minimization was performed using PRODRG online server. Energy values were obtained for all three ligands by Hex docking software. Docking structure was visualized by Qmol software. H-bond interaction and length visualization was done by SWISS PDB VIEWER. The docking results suggested that 20-hydroxy ecdysone had lowest docking energy and thus highest affinity and hence, could be used for

receptor mediated drug delivery system against *Monodon baculovirus* in black tiger shrimp.

Sivakamavalli et al. (2014) performed homology modelling, molecular dynamics, and docking studies of *Fenneropenaeus indicus* lipopolysaccharide b-1.3 glucan-binding protein (LGBP), a pattern-recognition transmembrane protein having an important role in immune mechanism of crustaceans. In this study, the homology model was constructed by using Modeller 9v8 based on X-ray structure for the same protein in *Thermotoga maritima*. This structure was further prepared using protein preparation wizard of Glide (Grid-Based Ligand Docking With Energetics). In this preparation after ensuring chemical accuracy, hydrogen was added to the structure at most likely positions and side chain conformation was optimized. The model validation was done using PROCHECK, PROSA and PyMol. The active site of predicted model was identified using SiteMap program. β -glucan was selected as ligand for docking and this ligand was prepared using LigPrep 2.5 which gives a number of structural outputs with various chemical features. β -glucan ligand was docked using Glide software from Schrodinger into the active site of LGBP. By adopting various refinement procedures, single best complex was generated. Molecular dynamic simulation was done for modeled LGBP and LGBP complex with β -glucan using Desmond model of Schrodinger. The results of above study indicate that Arg71 from cell adhesion domain of LGBP forms strong hydrogen bond with the ligand. It has also been suggested that Arg34, Lys68, Val135, and Ala146 are important residues as they have strong bonding interactions in the active site. In this study, yeast agglutination assay and phagocytosis assay were also done and the results indicate that LGBP possesses sugar binding and recognition site in its structure that are responsible for positive agglutination reaction.

Misra et al. (2013) performed homology modelling and docking studies of enzyme β -ketoacyl-ACP synthase III (FabH) involved in type II fatty acid biosynthesis of *Chlorilla variabilis*. The enzyme FabH was docked with its substrate acyl carrier protein (ACP). Homology modelling of FabH was done using

MODELLER 9v9 taking X-ray crystal structure of *E coli* FabH as template protein. The model was validated using PROCHECK, WHATCHECK, VERIFY3D, ERRAT, PSQS and PROSA. The active site of FabH was predicted by CASTp program. The docking of ligand ACP with FabH enzyme was performed by using Patch-Dock (rigid protein-protein docking server) and Fiber-Dock (Flexible induced-fit backbone refinement in molecular docking). The results were further refined by Fiber-Dock. Molecular dynamic (MD) simulation was also performed using GROMACS (version 4.0.5 software). The active site of acetyl-CoA was predicted as a cavity made up of 53 amino acids in the form of 20 Å long hydrophobic tunnel, located between the core β sheets and α helices of FabH. The predicted binding site for ACP is the electropositive surface surrounding the active site tunnel. Various positively charged and hydrophobic residues significantly interact with ACP through H-bonding.

3. MATERIALS AND METHODS

3.1. Site of Experiment

The research work was carried out in the Molecular Biology laboratory of Fish Genetics and Biotechnology Division, Central Institute of Fisheries Education (Mumbai), and Centre for Agricultural Bioinformatics, Indian Agricultural Statistical Research Institute (New Delhi).

3.2. Sample Collection

Clarias batrachus specimens of ~150g were collected from CIFE Balabhadrapuram station of Kakinada Centre of CIFE. The fish were anaesthetized using clove oil (50µl/ l water) and aseptically dissected for the collection of brain, testis and ovary tissues. The tissues were stored in RNAlater™ solution (Qiagen, Netherlands) and transported to Fish Genetics and Biotechnology lab, CIFE, Mumbai. The samples were stored at -80°C until RNA isolation.

3.3. Equipment and Laboratoryware

The equipment used during the course of this study include thermocyclers from BioRad and Applied Biosystems, USA, Nanodrop spectrophotometer (Thermo, USA), refrigerated centrifuge (Thermo, USA), horizontal gel electrophoresis apparatus (Technosource, India), shaking incubator (ORBITEK, India), gel documentation system (BioRad, USA), tissue homogenizer (Tissue Tearor, USA) thermostatic water bath (Julabo, Germany), electronic balance (OHAUS, USA), tabletop centrifuge 5430 (Eppendorf, Germany), microwave oven (Kenstar, India), autoclave hot air oven (Newtronic, India) and micropipettes (Eppendorf, Germany). The glassware was sourced from Borosil, India and plasticware from Tarsons, India.

3.4. Chemical Reagents and Kits

Chemicals used in this study were generally of molecular biology grade. Agarose, ethidium bromide, Tris base, EDTA, chloroform, isoamyl alcohol, isopropanol, LB agar and LB broth were purchased from Himedia, India. Ampicillin was purchased from Sigma Aldrich. Ethanol was purchased from Merck, India. TRIzol® reagent was purchased from Invitrogen. 2X PCR master mix, nuclease free water, 6X gel loading dye, Xgal and IPTG were purchased from Fermentas, USA. The kits used in the study were Maxima First Strand cDNA Synthesis Kit (Fermentas, USA), QIAquick Gel Extraction Kit (Qiagen) and InsTAclone PCR cloning kit (Fermentas, USA). All the solutions were prepared in autoclaved demineralized water (DMW, Milli Q). All solutions, plasticware and glassware except heat labile components were sterilized by autoclaving at 15 lb for 20 min. Heat labile solutions were filter sterilized using 0.22µ cellulose acetate disposable syringe filters (Osmonics, USA). Details of reagent composition are provided in Annexure I.

3.5. Bacterial Strain and Vector

3.5.1. Bacterial strain

E. coli DH5α, a recombination deficient amber suppressing strain, available at Fish Genetics and Biotechnology Division, CIFE, was used as host strain. The genotype of DH5α is supE44 ΔlacU169 (φ 80 lac Z ΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1.

3.5.2. Culture conditions

Cultures were maintained on LB-agar plates stored at 4°C for regular use. They were transferred to fresh slants after every 20 days. For long-term storage, 1.5 ml aliquot of O/N culture was transferred to a 2 ml screw-cap tube and 0.5 ml of autoclaved glycerol (60%) was added. The tube was vortexed briefly to ensure complete dispersal of glycerol. The tube was snap frozen in liquid nitrogen and stored at - 80°C indefinitely. To recover the culture, frozen surface of the culture was scraped with a sterile pipette tip, streaked on a selective agar plate and incubated overnight at 37°C.

3.5.3. Plasmid vector

Plasmid pTZ57R/T vector provided along with InsTAclonePCR cloning kit (Fermentas, USA) was used for T/A cloning of the PCR amplified products. It has ampicillin resistance marker.

3.6. Enzymes

TaqDNA polymerase was purchased from Merck, India. T4 DNA ligase was provided with the InsTAclonePCR Cloning kit (Fermentas, USA). Reverse transcriptase and Ribolock RNase inhibitor were provided with the Maxima first strandcDNA synthesis Kit (Fermentas, USA). Proteinase K and RNase A were obtained from Fermentas, USA.

3.7. Nucleic Acids

Gene Ruler 100 bp Plus and 1 kb DNA ladders were purchased from Fermentas, USA. Custom synthesized primers were obtained from Xcelris Genomics Lab, Ahmedabad, India.

3.7.1. Total RNA extraction

Total RNA was extracted using TRIzol® reagent (Invitrogen, USA) following the manufacturer's instructions. Tissue sample (100 mg) from brain, testis and ovary was transferred to a 2.0 ml microfuge tube and homogenized in 1 ml Trizol™ reagent (Invitrogen, USA) using sterile forceps and tissue homogenizer (Tissue Tearor, USA) for 20-30 s at room temperature (r.t.). After incubating the homogenate for 5 min at r.t. to allow lysis, ~200 µl chloroform per ml of Trizol was added and shaken vigorously for 15 s and kept at r.t. for 5-15 min. After centrifugation at 12000g for 10-15 min at 4°C and the supernatant was collected without disturbing the interface and transferred to a fresh 1.5 ml tube. RNA was precipitated by adding 500 µl of ultra-pure isopropanol (0.5 vol/ ml Trizol™ reagent). The tube was vortexed for 5-10 s and incubated at r.t. for 5-10 min.

The contents were centrifuged at 12000g for 10 min at 4°C and the supernatant was discarded. One ml of chilled 70% ethanol (prepared in DEPC water) per ml of Trizol was added and again centrifuged at 7500g for 5 min. The ethanol was then removed and the pellet was air dried for 30 min. The pellet was suspended in 30-50 µl of nuclease free water/DEPC water (Annexure I). The quality of RNA was confirmed using Nanodrop spectrophotometer (Thermo Scientific, USA). Nucleic acid concentration was obtained directly in terms of nanogram per microliter along with the 260 : 280 ratios. The ratio of the absorbance at 260 nm and 280 nm provides an estimate of the purity of the isolated DNA/RNA. The integrity of RNA was checked on 2% agarose gels as described below. RNA samples were then stored at -80°C until further use.

3.7.2. Gel electrophoresis of RNA

The integrity of the isolated RNA was checked by electrophoresing on a 2% agarose gel. The gel casting tray and apparatus was soaked in 1% SDS for 1 h, washed with Milli Q water and finally rinsed with DEPC treated Milli Q water. Appropriate amount of agarose (0.8 g) was added to 40 ml of 0.5 X TAE buffer and melted in a microwave oven till the solution became completely clear. Ethidium bromide (final concentration of 5 µg/ ml) was added to the agarose solution cooled to about 60°C. This was poured into the gel tray and allowed to solidify. The comb was removed carefully after 30 min and the gel tray was placed in the gel electrophoresis chamber containing 0.5 X TAE buffer such that gel was just submerged in the buffer. Appropriate volume of RNA (1 µl) was mixed with 1 µl of 6X gel loading buffer and electrophoretic buffer and loaded into the gel slots. The unit was connected to the power supply and the gel was run at 80 V for 1 h. The gel was then taken out and documented in a Gel documentation unit (BioRad, USA).

3.7.3. RNA purification and complementary DNA synthesis

The RNA was treated with DNase I (RNase free; Fermentas, USA) to remove the genomic DNA contamination. A 10 µl reaction mix containing 2 µg RNA,

1 μ l DNase I buffer, 1 μ l DNase I and nuclease free water was prepared. The reaction was incubated at 37°C for 30 min. To terminate the reaction, 1 μ l of 25 mM EDTA was added and incubated at 65°C for 10 min.

The DNase I treated RNA was converted into single stranded cDNA using the Maxima First Strand cDNA Synthesis Kit (Fermentas, USA) as per the protocol provided by the manufacturer using oligo-dT as primer. Briefly, 1 μ l of oligo-dT, 1 μ l dNTP (2.5 mM each) and 2 μ l of nuclease free water were added to the 11 μ l reaction mix described above. It was mixed gently, incubated at 65°C for 5 min. Nucleic acid concentration was obtained directly in terms of ng/ μ l along with the and chilled on ice. Subsequently, 4 μ l RT buffer and 1 μ l Maxima RTase enzyme were added so that the total volume becomes 20 μ l. After gentle mixing the tube was incubated at 50°C for 30 min and the reaction was terminated by heating at 85°C for 5 min.

3.8. Primer Designing

3.8.1. Designing of degenerate primers for partial gene characterization

Degenerate PCR primers were designed to amplify partial sequences of *C. batrachus* reproductive genes, namely glycoprotein α subunit (GtH α), follicle stimulating hormone β subunit (FSH β), luteinizing hormone receptor (LH-R) and gonadotropin releasing hormone receptor (GnRH-R). Amino acid and nucleotide sequences of orthologs of these genes reported from taxonomically related fish species were retrieved from NCBI in FASTA format. Multiple sequence alignment of both amino acid and nucleotide sequences was carried out using CLUSTALW. Degenerate primers were designed for GtH α and FSH β based on conserved amino acids and used for partial amplification. For LH-R and GnRH-R genes, the degenerate primers were designed based on conserved nucleotide sequences.

3.8.2. Primer designing for RACE

Specific primers were designed for the amplification of flanking regions of the partial sequences of *C. batrachus* GtH α , FSH β , LH-R and GnRH-R genes. As per the 3' and 5' Rapid Amplification of cDNA Ends (RACE) protocol, two gene specific primers, GSP1 and GSP2 (nested) were designed for amplification of both the ends using software Generunner V. 3.0. Several factors, such as primer length, %GC content, melting temperature, annealing temperature, secondary structures, etc. were taken in to consideration while designing the primers. The basic features of the primer included a length of 18 to 22 nucleotides, 40-60% GC content, 55 - 65°C annealing temperature, lack of internal secondary structures and lack of complementarities at 3' end to prevent primer dimer formation. Primers were synthesized commercially by Xcelris Genomics Ltd., India. The primers received in lyophilized form from the supplier were dissolved in sterile TE buffer (pH 7.0) to get a stock solution of 100 pmol/ μ l concentration. The stock solution was then diluted to get a working concentration of 10pmol/ μ l with sterile DMW.

3.9. PCR Amplification

The cDNA synthesized from brain and testis tissue samples was used for PCR amplification of desired sequences. For GtH α , FSH β and GnRH-R cDNA prepared from brain tissue was used as template while for LH-R cDNA was prepared from testis tissue. PCR was done in a reaction volume of 25 μ l containing 2.5 μ l (1X) *Taq* polymerase buffer, 2 μ l of 2.5 mM dNTPs, 1 μ l of 10 pmol forward primer, 1 μ l of 10 pmol reverse primer, 1 μ l of cDNA, 0.5 μ l of 3U/ μ l *Taq* DNA polymerase. The volume was made up with nuclease free water. Touchdown PCR was performed with initial denaturation at 94°C for 3 min, 35 cycles of 94°C for 30 s, touchdown annealing conditions specific to the primers for 30 s, extension at 72°C for 45 s and 1 cycle of final extension at 72°C for 5 min.

3.10. DNA Recovery from Agarose Gel

The PCR products were separated on 1.5% agarose gels and the

desired DNA fragments were sliced carefully from the agarose gel with a cover slip avoiding excess agarose. DNA was eluted using QIAquick Gel Extraction Kit (Qiagen) following manufacturer's instructions and DNA was collected in 1.5 ml microfuge tubes in volumes ranging from 10 to 30 µl depending on the band volume. The eluted product was quantified on Nanodrop spectrophotometer (Thermo Scientific, USA)

3.11. T/A Cloning of Amplicons

PCR products amplified by *Taq* polymerase have an extra single 3'-A overhang added to each strand of the PCR product. This allows cloning into vectors that have been specifically designed to carry a 3'-T overhang on the complementary strand (called T/A vectors). In the present study, the desired DNA fragments were cloned into pTZ57R/T vector and transformed into *E. coli* DH5α strain using Ins TAclone™ PCR Cloning Kit (Fermentas, USA) following manufacturer's instructions. Ligation was done in a 15 µl reaction that was prepared by adding 1.5 µl pTZ57R/T vector (0.09 pmole ends), 3 µl of 5X buffer, 0.27 pmole ends of the insert (calculated based on insert size using a chart provided by the manufacturer), 1 µl T4 DNA ligase enzyme and nuclease free water to raise volume up to 20 µl. The mixture was incubated at 16°C overnight and stored at -20°C until transformation.

3.12. Transformation

For transformation, 150 µl of overnight LB broth culture of DH5α was used to inoculate 1.5 ml of C medium (provided in the Ins TAclone™ PCR Cloning Kit; Fermentas, USA). After 30 min incubation at 37°C, cells were harvested by centrifugation at 12000 rpm for 3 min. The supernatant was discarded and the pellet was suspended in 300 µl "T" solution (provided in kit). After keeping it on ice for 5 min the cells were harvested and the supernatant discarded. The pellet was suspended in 120 µl of T solution followed by incubation on ice for 5 min. To the cell suspension, 2.5 µl of the ligation mix was added and incubated on ice for 5 min. This was then spread on Xgal-IPTG-Ampicillin LB agar plates (Annexure I) and kept for

incubation overnight at 37°C. The white colonies were further taken for screening.

3.13. Confirmation of Positive Clones

White colonies were screened for the presence of insert by colony PCR using gene specific primers. A few cells from each colony were picked and suspended in 10 µl TE using a sterile tip. Two µl of this suspension was used as template in a PCR reaction that was set up as described above. The PCR products were separated on 1% agarose gel and the insert sizes were confirmed by using Gene Ruler 100bp Plus ladder.

3.14. Sequencing of Clones

Plasmid DNA was isolated from positive colonies using QIAprep Spin. Miniprep Kit (Qiagen, USA), following manufacturer's instructions. The plasmid was quantified using Nanodrop spectrophotometer and visualized on 1% agarose gel. The samples were sequenced commercially by Xcelris Genomics Ltd. (India) using universal M13 primers and Sanger's sequencing method. The sequences obtained were confirmed by using NCBI BLAST homology tool

3.15. Rapid Amplification of cDNA Ends (RACE)

The full-length cDNA sequence was obtained by amplification of cDNA ends using RACE (Rapid Amplification of cDNA Ends) technique. RACE is a procedure for the amplification of nucleic acid sequences from a messenger RNA template between a defined internal site and either the 3' or 5' end of the mRNA. Hence, it is called 5'-RACE or 3'-RACE depending on which end of the mRNA is amplified. This methodology of amplification with single-sided specificity has been described by others as "one-sided" PCR or "anchored" PCR. In this study both 5' and 3' RACE were performed using 5' and 3' RACE kit (Life Technologies, USA). Full length cDNAs that are synthesized by this technology have a patented 5' RACE abridged anchor primer for amplification of dC tailed cDNA and an oligo(dT) adapter primer bound to the 3' end and abridged universal amplification primer.

3.15.1. Protocol for 3' RACE

This takes advantage of the natural poly-(A) tail found in mRNA as a generic priming site for PCR. In this procedure mRNA are converted into cDNA using reverse transcriptase (RT) and an oligo-dT adapter primer. Specific cDNA is then amplified by PCR using a gene specific primer (GSP) that anneals to a region of known exon sequence and an adapter primer that targets the poly (A) tail region. This permits the capture of unknown 3'-mRNA sequences that lie between the known mRNA sequence and the poly (A) tail. The detailed procedure of 3' RACE is given in Fig. 1.

3.16.2. First strand cDNA synthesis for 3' RACE

For 3'RACE, first strand cDNA synthesis was initiated at the poly (A) tail of mRNA using the adapter primer (AP) and the reaction was catalyzed by SuperScript™ II RT as per the protocol of 3' RACE kit (Invitrogen, USA), supplied by the manufacturer. The reaction mixture was incubated initially at 42°C for 50 min and additionally at 70°C for 15 min. After first strand cDNA synthesis, the template mRNA was destroyed with RNase H enzyme, which is specific for RNA: DNA hetero duplex complex.

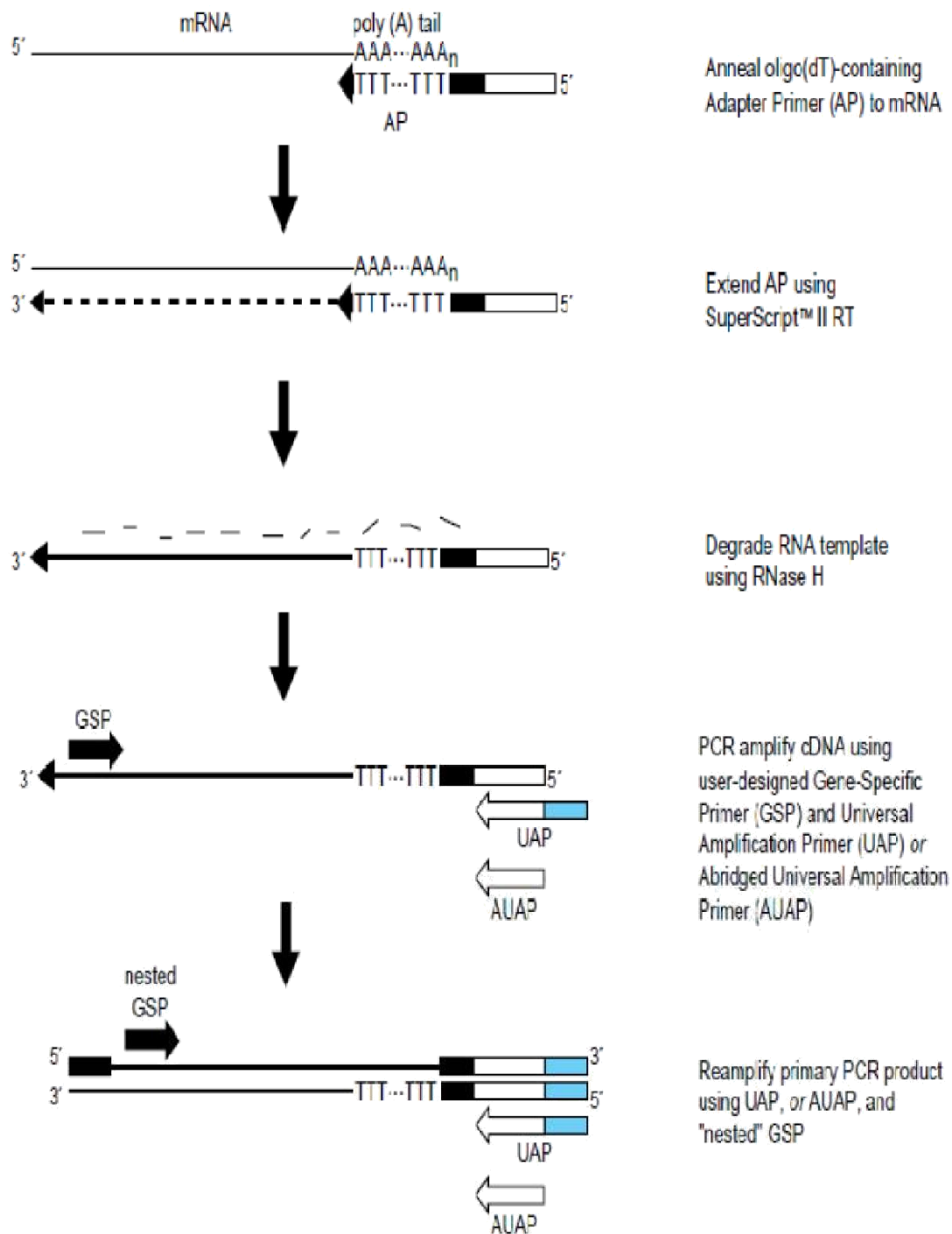


Fig. 1. Schematic representation of 3' RACE protocol

The reaction mixture for 1st strand cDNA synthesis for 3' RACE was prepared as follows:

Total RNA	:	1-5 µg
10 µm AP solution	:	1 µl
DEPC treated water	:	To make up volume to 12 µl

The reaction mixture was incubated at 70°C for 10 min followed by chilling on ice for at least 1 min. The following reagents were added after a brief spin.

10x PCR buffer	:	2 µl
25 mM MgCl ₂	:	2 µl
10 mM dNTP mix	:	1 µl
0.1 M DTT	:	2 µl

After mixing, the tube was equilibrated to 42°C for 2 to 5 min, 1 µl of SuperScript™ II RT was added followed by incubation at 42°C for 50 min. The reaction was terminated by incubating at 70°C for 15 min followed by chilling on ice. The tube was briefly spun and 1 µl of RNase H was added followed by incubation at 37°C for 20 min.

3.15.3. PCR amplification for 3' RACE

PCR amplifications were performed as described by Sambrook and Russel (2001). The amplification reaction was carried out in 25 µl final volume in 0.2 ml PCR tubes in a heated lid thermocycler. To reduce the nonspecific binding and the extension of primers during the initial denaturation process 'hot-start' practice was performed. The PCR conditions were as described above.

3.16. Protocol for 5' RACE

First strand cDNA synthesis was primed using a gene-specific antisense oligonucleotide (GSP1). This permits cDNA conversion of specific mRNA, or related families of mRNAs, and maximizes the potential for complete extension to the 5'-end of the message. Following cDNA synthesis, the first strand product was purified from unincorporated dNTPs and GSP1. TdT (Terminal deoxynucleotidyl transferase) is used to add homopolymeric tails to the 3' ends of the cDNA. Tailed cDNA is then amplified by PCR using a mixture of three primers: a nested gene-specific primer (GSP2), which anneals 3' to GSP1; and a combination of a complementary homopolymer-containing anchor primer and corresponding adapter primer which permit amplification from the homopolymeric tail. This allows amplification of unknown sequences between the GSP2 and the 5' end of the mRNA. The detailed procedure is given in Fig. 2.

3.16.1. First strand cDNA synthesis for 5' RACE

First strand cDNA was synthesized from total RNA using a gene-specific primer (GSP1) and SuperScript™ II, a derivative of Moloney Murine Leukemia Virus Reverse Transcriptase (M-MuLV RT) with reduced RNase H activity as per the protocol of 5' RACE system kit (Invitrogen, USA). For 5' RACE, the first strand cDNA was synthesized from 2.5 µg total RNA. After first strand cDNA synthesis, the original mRNA template is removed by treatment with the RNase Mix provided in the kit.

Reaction mixture for 1st strand cDNA synthesis for 5' RACE was prepared as follows:

GSP1	:	2.5 pmoles (~10 to 25 ng)
Total RNA	:	1-5 µg
DEPC-treated water	:	To make up volume to 15.5 µl

The tube was incubated at 70°C for 10 min to denature the secondary structures of RNA followed by chilling on ice for 1 min.

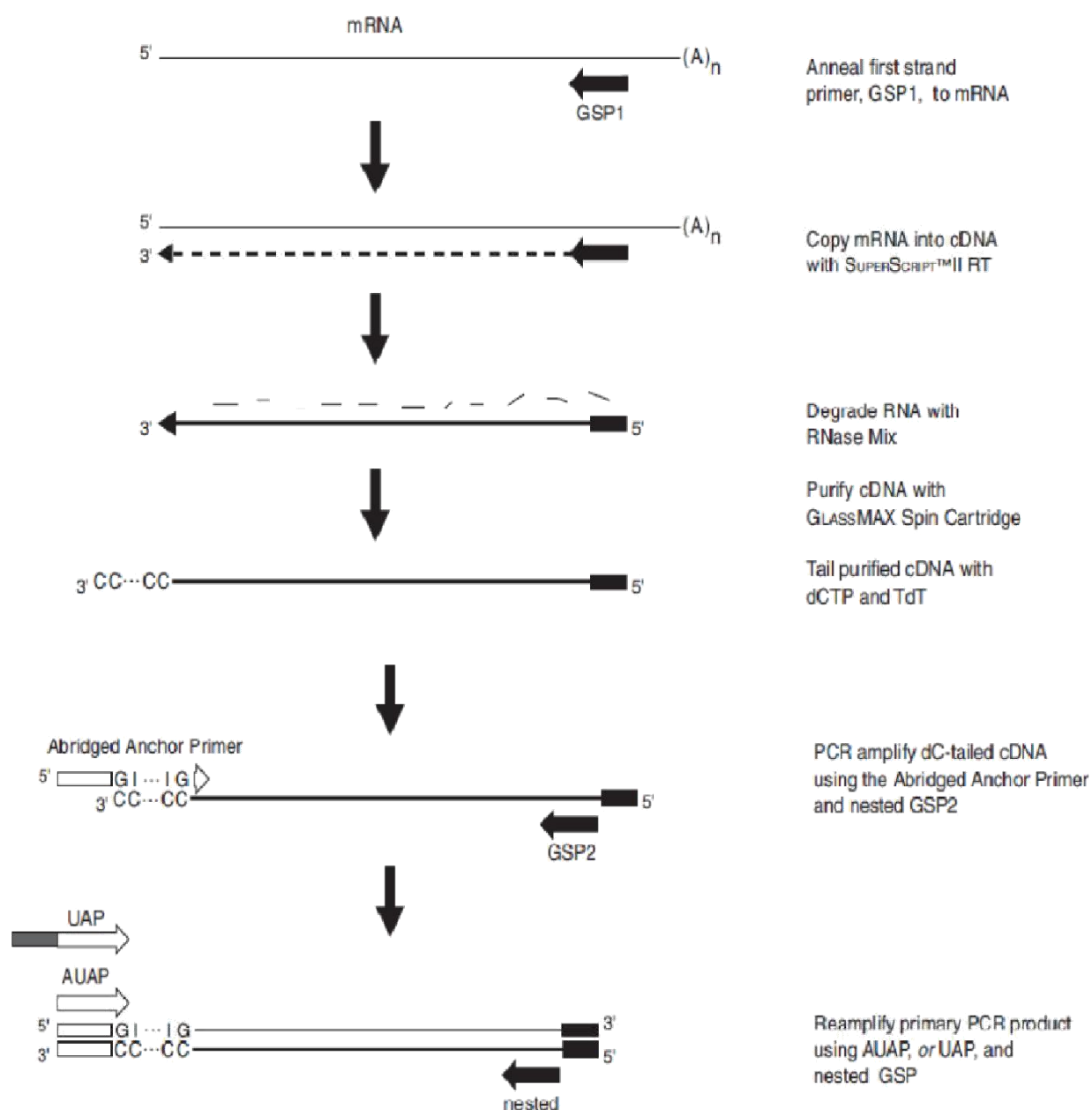


Fig. 2. Schematic representation of 5' RACE protocol

After a brief spin the following reagents were added to the tube in the order given.

10X PCR buffer	:	2.5 μ l
25 mM MgCl ₂	:	2.5 μ l
10 mM dNTP mix	:	1 μ l
0.1 M DTT	:	2.5 μ l

The contents were mixed properly before a spin, tube was incubated at 42°C for 1 min and 1 μ l of SuperScript™ II RT was added followed by incubation at 42°C for 50 min. The reaction was terminated by incubating the tube at 70°C for 15 min. To degrade the RNA template, 1 μ l of RNase H mix was added to the tube, mixed gently but thoroughly and incubated at 37°C for 30 min.

3.16.2. Purification of first strand cDNA

Excess nucleotides and GSP1 must be removed from the first strand cDNA product before PCR. Otherwise, residual GSP1 would be tailed by TdT and compete for Abridged Anchor Primer during PCR. Therefore, the first strand cDNA product was purified by S.N.A.P column provided with the 5' RACE system kit (Invitrogen, USA) following suppliers guidelines. Briefly, 120 μ l of binding solution (6 M NaI) was added to the first strand reaction, mixed properly and transferred to the S.N.A.P. column. The column was centrifuged at 13,000xg for 20 s, flow through was discarded and transferred the column to a fresh tube. TO the column, 0.4 ml of cold (4°C) 1X wash buffer was added, centrifuged at 13,000xg for 20 s and flow through was discarded. This step was repeated for 3 more times. The column was washed with 400 μ l of cold (4°C) 70% ethanol twice. After discarding the flow through an additional spin at 13,000 x g for 2 min was given to remove any residual ethanol from the column. After that, the column was transferred into a fresh 1.5 ml microfuge tube and the DNA was eluted in 50 μ l of sterile distilled water (preheated to 65°C) by centrifuging at 13,000xg for 20 s.

3.16.3. Poly (C) tailing of first-strand cDNA

TdT tailing creates the abridged anchor primer binding site on the 3'-end of the cDNA. The purified PCR product was subjected to homopolymeric C-tailing to the 3' end of first-strand cDNA using recombinant terminal transferase and dCTP. The reaction mixture contained the following in a total volume of 25µl:

Purified cDNA sample	:	16.5 µl
5X tailing buffer	:	5 µl
2 mM dCTP	:	2.5 µl
DEPC-treated water	:	To make up volume to 25 µl

The reaction mixture was thoroughly mixed and briefly spun followed by incubation at 94°C for 3 min and immediate chilling on ice for 1 min. To this, 1 µl of terminal transferase (TdT) was added and incubated at 37°C for 20 min. Finally, the mixture was incubated at 65°C for 10 min to heat inactivate the terminal transferase enzyme.

3.16.4. PCR amplification of dC-tailed cDNA

The PCR was performed using 2.5 µl of the dC-tailed cDNA as a template with abridged anchor primer (AAP) supplied with the 5' RACE kit (Invitrogen, USA) and gene specific primer 2 (GSP2). Touch-down PCR was performed for the amplification of targeted cDNA to reduce mispriming. PCR conditions included an initial denaturation of 95°C for 3 min followed by 30 cycles of 94°C for 30 s, annealing from 60-55°C for 30 s and extension at 72°C for 60s with a final extension of 10 min at 72°C. After confirmation in agarose gel electrophoresis, the PCR product was gel purified using QIAquick gel extraction kit (Qiagen, Netherlands) and T/A cloned into pTZ57R/T vector using InsTAclone PCR cloning kit (Thermo Scientific, USA) following manufacturer's guidelines.

3.17. Sequence Analysis

Online bioinformatics' tools and software were used for analysing amplified cDNA sequences. BLASTn program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) was used to find homologous sequences from closely related organisms. Multiple sequence alignment and Neighbor-joining tree analysis were performed by MEGA 6.0 software.

3.18. Homology Modeling and Docking

In homology modeling an atomic model is built based on experimentally determined structure of a protein having similar amino acid sequence to the target. BLASTp search was done to select best templates with high homology to *C. batrachus* FSH β (this study), LH β (Accession AIU98447.1) proteins and *Clarias gariepinus* FSH-R (Accession CAB51907.2), LH-R (Accession AAN75752.1), FSH β (Accession AAN75753.1), LH β (Accession CAA66359.1), GnRH-R1 (Accession CAA66128.2), GnRH-R2 (Accession AAM95605.1) and salmon GnRH-R4 (Accession AHL26526.1) proteins. Homology modeling was also done for active GnRH decapeptides of salmon (Accession CAA56148.1), catfish (Accession. CAA54971.1) and chicken (Accession. CAA54969.1).

Target-template realignments were achieved by BLASTp. Homology modeling was done by MODELLER 9.14 (Andrej Sali lab) which performs comparative protein modeling by satisfying various spatial restraints. In this process spatial restraints from the template are transformed to the target. The 3D model is obtained by satisfying all the restraints. Energy minimization of all models was done to minimize unfavorable contacts using Discovery Studio 4.1 (Accelrys Enterprise Platform) using CHARM force field. The stereochemical quality of the model was verified by using Procheck v.3.5 (European Bioinformatics Institute). Ramachandran plot was visualized using Swiss-PdbViewer v.4.1 (Swiss institute of Bioinformatics). Receptor hormone docking has been performed using PatchDock online server (<http://bioinfo.3d.cs.tau.ac.il/PatchDock>). This server performs rigid protein-protein

docking based on shape complementarity criteria. The outputs of PatchDock were refined using flexible docking server FireDock (<http://bioinfo.3d.cs.tau.ac.il/FireDock>).

4. RESULTS

4.1. PCR Amplification of Targeted Genes

Degenerate primers were designed for PCR amplification of four reproductive genes of *Clarias batrachus* including gonadotropin hormone alpha subunit (GtH α), follicle stimulating hormone β subunit (FSH β), luteinizing hormone receptor (LH-R) and gonadotropin releasing hormone receptor (GnRH-R; Table 1). Total RNA was isolated from brain and testis tissues of mature *C. batrachus* males (Fig. 3). Characteristic 28S, 18S and 5.8S rRNA bands were visible. DNase I treated RNA was used for preparing cDNA that served as template for PCR.

Complementary DNA prepared from brain tissue was used as template to amplify partial fragments of GtH α , FSH β and GnRH-R transcripts, while cDNA from testis was used for amplification of LH-R. Amplicon sizes of 201, 213, 342 and 1064 bp were obtained for GtH α , FSH β , GnRH-R and LH-R, respectively (Fig. 4a – d). The bands were TA cloned in pTZ57R/T plasmid vector and commercially sequenced with M13 universal primer used methods described earlier (Section 3.7 to 3.14).

4.2. Sequence Analysis

BLASTn homology search (<https://blast.ncbi.nlm.nih.gov>) was performed with the obtained sequences. *C. batrachus* GtH α sequence showed 95% sequence identity to the *Clarias gariepinus* ortholog with 84% coverage. The FSH β had 98% identity with 95% coverage to the *C. gariepinus* sequence. A sequence identity of 89% to *Ictalurus punctatus* with 97% coverage was observed for LH-R. The GnRH-R sequence once again showed highest similarity of 96% to the *C. gariepinus* gene although the sequence coverage was only 36%. The complete GnRH-R transcript reported for *C. gariepinus* is about 2 kb while only 342 bp of the *C. batrachus* gene was amplified by degenerate primers due to consensus region constraints.

Table 1. List of primers designed

Sr. No.	Name	Primer sequence	Annealing temperature
1	GtHa degF	TAYCAGTGYATGGMTGCTG	55 – 50°C
2	GtHa degR	CARTGGCAGTCTGTRTGRTT	55 - 50°C
3	FSH β degF	GARTGTGGSAGCTGCRTCAC	55 - 50°C
4	FSH β degR	CAYTCACAGCTSAGRGCNAC	55 - 50°C
5	LH-R degF1	GAYGCNTTYAAYCCNTG	55 - 50°C
6	LH-R degR1	ATNGTRTGCCANCKYTC	55 - 50°C
7	GnRH-R degF1	TGGAAYRTIACIGTICARTG	55 - 50°C
8	GnRH-R degR1	RAACCARTACCAIAYICC	55 - 50°C
9	GnRH-R degF2	TGGAATKTSACRGTSAGTGG	55 - 50°C
10	GnRH-R degR2	TGGAACCARTACCAGATNCC	55 - 50°C
11	LH-R degF2	CTGAGCATMTCNAAYACKGG	55 - 50°C
12	LH-R degR2	TCYTCRCABGGRTTAAHGC	55 - 50°C
13	LH-R degF3	CTGAGATACCTGAGCATCTGCAACAC	55°C
14	LH-R degR3	GCATGAGTGATGGTGTGCCAGCGCTC	55°C
15	GnRH-R degF3	TGGCAGGAAACGGCATACAACATGTT	55°C
16	GnRH-R degR3	CCGAGGAGGTAATACGGCGTCCAGCA	55°C
17	FSH β 3'RACE	TCGTCCTTCACCTACCCTGTGG	60°C
18	GTH α 3'RACE	TGTTGCCAAAGAGGTTAAACGG	60°C
19	LH-R 3'RACE1	TTGCTGCTTATCGCTTTGGC	55°C
20	LH-R 3'RACE2	ACGCTATGGACTGGCAGACG	55°C
21	GnRH-R 3'RACE1	TGTGTTTCCTCTGCTCGTCATG	55°C
22	GnRH-R 3'RACE2	TTGCCTGAGACGCAGTGGAACG	55°C
23	FSH β 5'RACE1	TACTCGGCTGCATGCTGAAGGC	55°C
24	GTH α 5'RACE1	AGCAAGTGCTGCAGTGGCAGTC	55°C
25	FSH β 5'RACE2	AGTGTTGATGGTGACGCAGCTC	55°C
26	GTH α 5'RACE2	CCCGTTTAACCTCTTTGGCAAC	55°C
27	LH-R 5'RACE1	ACGGAGAGCGGTGGATGATAC	55°C
28	LH-R 5'RACE2	GCTTTGGATATGCCTTGAAACG	55°C
29	GnRH-R 5'RACE	AAGCGTGACAAAGTGGAACATG	55°C
30	GnRH-R 5'RACE	GGAGGCGACGATGATGATGGTC	55°C



Fig. 3. Total RNA extracted from *C. batrachus* tissues.
Lane 1: Brain; L 2: Testis

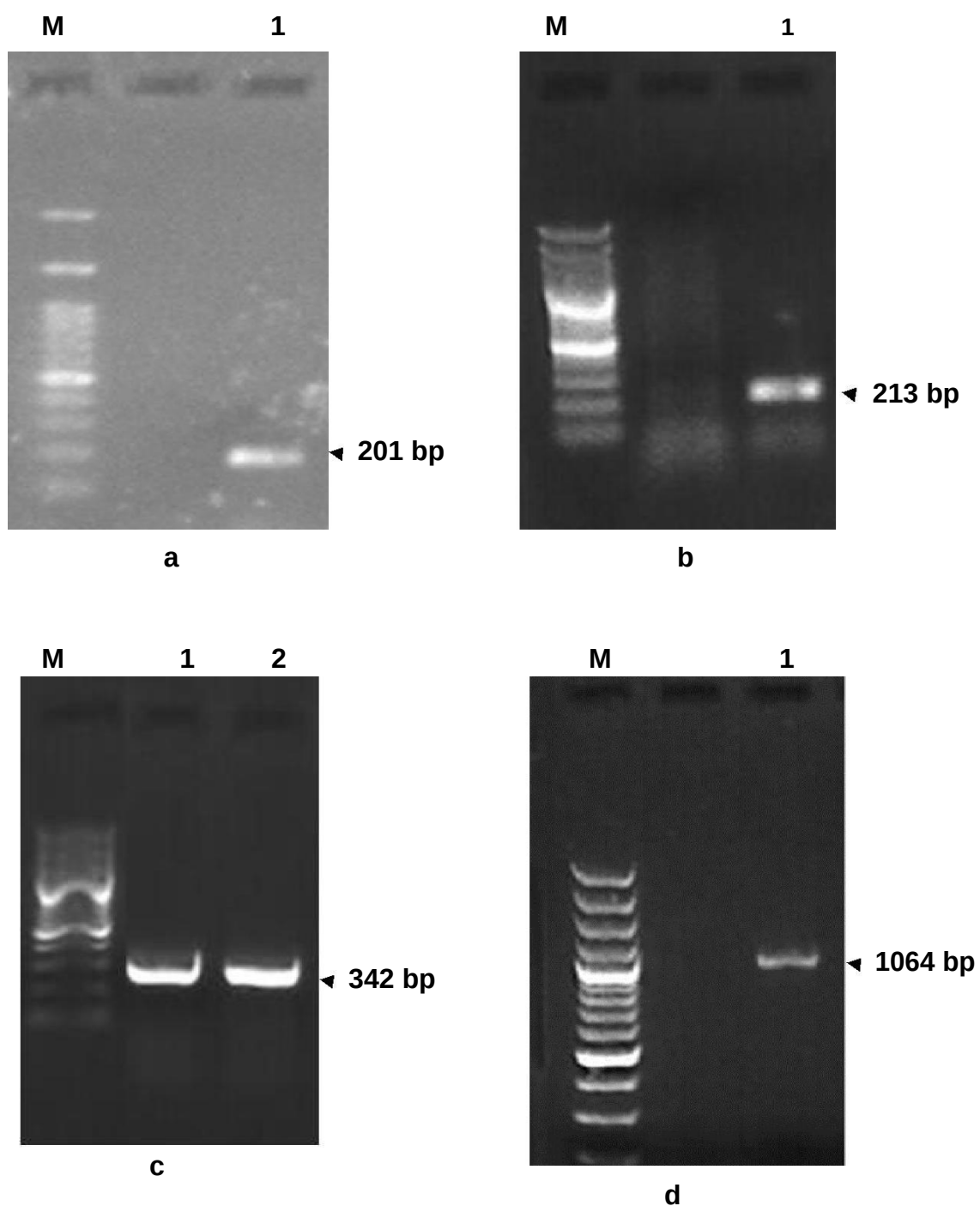


Fig. 4a - d. PCR amplification partial gene fragments. (4a) Lane M: 100 bp ladder GeneRuler Plus (Fermentas, USA), L1: GtH α amplicon; **(4b)** Lane M: As above, L1: FSH β amplicon; **(4c)** Lane M: As above, L1&2: GnRH-R amplicons **(4d)** Lane M: As above, L 1: LH-R amplicon

4.3. RACE for Determining 5' and 3' Ends

To determine the 5' and 3' ends of the transcripts by RACE, gene specific primers were designed for all the partial sequences and used with the universal amplification primer (AUAP) provided in the RACE kit (Life Technologies, USA). Nested gene specific primers were also designed for confirmation of the amplicons (Table 1). For GtH α , a 350 bp product could be amplified with GtH α -3'RACE and AUAP forward and reverse primers (Fig. 5a). The amplicon was purified from 1.5% agarose gel, TA cloned and sequenced as described earlier. Analysis revealed additional 274 bp of the 3'-region of the GtH α gene. For FSH β , a 500 bp amplicon was obtained with FSH β -3'RACE and AUAP primers (Fig. 5b). Sequence analysis revealed 456 bp of additional 3' information. BLASTn homology search with the now extended GtH α and FSH β sequences revealed 91% identity with the respective genes of *C. gariepinus* and the sequence coverage was 91% for FSH β and 89% for GtH α transcripts. All the sequences generated in this work are given in Fig. 6.

All the other gene specific primers designed for 3' end extension of LH-R and GnRH-R, along with 5' RACE primers for all the four genes failed to amplify desired sequences.

4.4. Homology Modelling and Docking Studies

The active decapeptide domain of *C. batrachus* and *C. gariepinus* is identical. However, it is salmon GnRH formulations that are used in the field for induced breeding (Fig. 7). Hence, homology modelling and docking studies of the three types of GnRH decapeptides salmon, chicken and catfish were done with the salmon (*Salmo salar*) GnRH-R and two receptors from *C. gariepinus* GnRH-R1 and R2. The complete *C. batrachus* GnRH-R sequence. In addition, homology modelling and docking studies of *C. batrachus* and *C. gariepinus* FSH β and LH β were done with homologous and heterologous receptors. Details of all the gene sequences used are provided in Table 2.

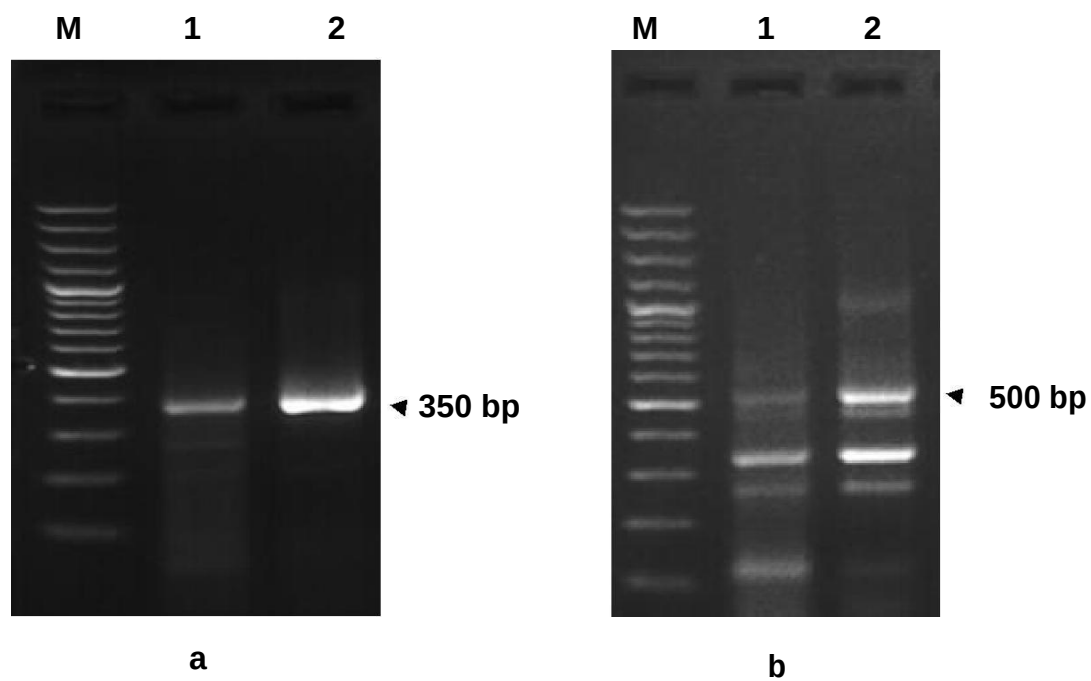


Fig. 5a & b. PCR amplification of 3' ends by RACE. (5a) Lane M: 100 bp ladder GeneRuler Plus (Fermentas, USA), Lanes 1 & 2: GtH α amplified fragments; **(5b)** Lane M: As above, L 1 & 2: FSH β amplified fragments (500 bp was the expected size)

>*Clarias batrachus* FSH gene partial cds

GAGTGTGGGAGCTGCGTCACCATCAACACTACAGCCTGTGCCGGCCTCTGTCAGACTCAGGAAAGAGCGTACCGCAGTCCAAT
GGCGCCATATTTCCAGAACACCTGTAACCTCAGGGATTGGACTTATGAGACCGTTTCAGCTGCCAGGCTGTGCTCCGGGGGTTG
ACTCGTCCTTCACCTACCCTGTGGCTCTGAGCTGTGAGTGCAGCCAGTGTAAACACAGAGATCACAGACTGCGGCGCCTTCAGC
ATGCAGCCGAGTAGCTGCCACACACACGCCTACTACTGAACCGAGTCACATCAGCTCAGCCAACAGCCCGGAGCTCTCACTGC
AGCAATCTGTTACACTTCTTTACGCATATTATAATTATTTTGAAATATTGCATCCTGTCAGGTTCCATCATTTACAATAATTATCC
TATCTCACCGTTTACACTTTACAAAGACCTACAAATATTTGTTTAAAAAAAACACACACACACACACACACACACACATACAGT
TCAATGCCTTATACTACCAATTAACCTCAGGTCTGTTGTGTTTGTCTGTTTGACCCGTGAACATCATGAAGGACATGATGCGCTGT
TGTTTAGCCTCACCTGAGCATGTAAACACTGCTTCATTAACACAGCTCACATGCAAAAAAAAAAAAAAAAAA

>*Clarias batrachus* GTH α gene partial cds

ATCAGTGATGGGCTGCTGCTTTTCCAGAGCTTACCCAACTCCTTTGAGATCCAAGAAAATATGCTGGTCCCAAAGAATATCAC
ATCTGAAGCAACATGTTGTGTTGCCAAAGAGGTTAAACGGGTATTGTTAATGACGTAAAGCTAGTGAATCACACAGACTGCCA
CTGCAGCACTTGCTACTATCATAAATTTAGATGCCATGACTTCATCATTTTACTAGTCTGTTCAAGAAATCCCTTAAACGGAAAT
CACACAGCAAAAAAAGTCTTTGTTTCATGTGTTGCTCAAAGTTCTGTTTATAATGTCTGTTATTAAGCTATATCCGCTTCTCTGA
TTAGTTTAAAACTTAAACCAATCTGCTAAGTGTAATTTGCCATTGTAATGTGACTTCAAATAAAGCATATGCTGCATCTAAAAA
AAAAAAAAAAAAAAAAAAAA

>*Clarias batrachus* LH receptor partial cds

CTGAGATACCTGAGCATCTGCAACACAGGAATAAGACATTTCCCGGATTTTTCTCGCATCTCCTCAATGGGGATGGATTTCTTTT
TGGAGATGGGAGACAACACACATCTAAACAGCATCCCAGCTAATGCGTTTCAAGGCATATCCAAAGCAGACATGTACATGAAAC
TTGTGAGAAATGGAATTACCGAGATCGAAGCCACGCCTTCAATGGGACCAGACTAGAAAAGTTGATCCTTAAAAACAACCAGC
ATCTAATTAATAATCCATGATGATGCGTTTGAGGGGGCCGAAGGACCACTTTACTTGATGTATCATCCACCGCTCTCCGTTCTGCT
CCCTACTCGAGGACTGGAAGAGATGAAGGTTCTCATAGCCCCGCTCTACACCTTCCCTGAAGACCCCTCCCTCCGCTGGAGAACC
TCTCAGAGCTGCAGGAGGCCAATCTACCTACCCAAGCCACTGCTGTGCCTTCCACACCTGGAACCGGACAACAAGGAGGCC
GCTGTATTTAGTTCGATTCAAGGATCTGACCATGCTGTGTAAACATGGCTGCCAAGCAGATACCATGGACCCATCGGGTGAAGGT
CTTAACGACATCAATTTCCACTACCCCGATCTGGAGTTGTGCGTCAGCGGCAGCTCCTTCAAGTGACCCCCAGAACCCGGACGC
CTTCAGTCCTTGCGAGGACTTGCTAGGGCAGAGTTTTCTGCGCGTCATCACTTGATTGTCACGACTTTCGCTCTCGTGGGAAA
CCTGGCCGTGCTGTTTGCCTGCTGCTGAGCGACCAGAAGCTGAGCGTCTCCCGGTTCTCATGTGCAACTTGGCCTTCGCGG
ATCTGTGCATGGGGTTCTATTTGCTGCTTATCGCTTTGGCGGACTATCGCTCGCGTCGAGAGTACTACAACCACGCTATGGACT
GGCAGACGGGCCTGGGATGTGGCGTAGCTGGGTTCTTGACTGTCTTCTCAAGCGAACTGTCGATCTATACTCTCACTATGATCA
CCCTAGAGCGCGGCACACCATCACTCATGC

>*Clarias batrachus* GnRH receptor partial cds

TGGCAGGAAACGGCATAACAATGTTCCACTTTGTACGCTTTATGTGTTTCTCTGCTCGTCATGAGCTTGTGCTATACACGTA
TCCTGATTGAAATCAACCGGCAAATGCACAGAAGTAAGGACAAAGGTGAGCATCTCTCTGATATGGTCATGTACTTGACATAC
CCTAAGTGTAATACTGTATATGTCTCTCCAGCAGGAGAGCCTTGCTGAGACGCAGTGGAACGGATATGATCCCCAAAGCTC
GTATGAAGACCCGTGAAATGACCATCATCATCGTCGCCTCCTTTGTCATTGCTGGACGCCGTATTACCTCCTCGG

Fig. 6. Nucleotide sequences of *C. batrachus* FSH, GTH α , LH-R and GnRH-R partial cds

CAA54969.1_chicken_GnRH_II	GHWSHGWYPG	10
CAA56148.1_S.salar_GnRH	GHWSYGWLPG	10
AIU98448.1_C.batrachus_GnRH	QHWSHGLNPG	10
CAA54971.1_C.gariepinus_GnRH	QHWSHGLNPG	10
	***.*	**

Fig. 7. Clustal alignment of active GnRH decapeptides showing perfect identity of *C. batrachus* and *C. gareipinus* sequences

Table 2. Template and query proteins used in study

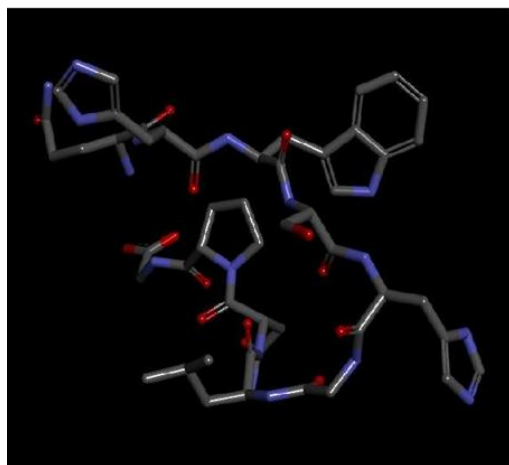
Sr.No.	Query protein	Template pdbID	Template name	Query cover (%)	Identity (%)
1	<i>C. batrachus</i> FSH β	1XWD	Human follicle stimulating hormone	76%	48%
2	<i>C. gariepinus</i> FSH β	1XWD	Human follicle stimulating hormone	76%	49%
3	<i>C. batrachus</i> LH β	1HCN	Human chorionic gonadotropin	79%	45%
4	<i>C. gariepinus</i> LH β	1HCN	Human chorionic gonadotropin	79%	45%
5	<i>C. gariepinus</i> FSH-R	4AY9	FSH complex with ectodomain of the receptor	48%	41%
6	<i>C. gariepinus</i> LH-R	4AY9	FSH complex with ectodomain of the receptor	48%	39%
7	Catfish GnRH	1YY1	LHRH synthetic construct	90%	80%
8	Chicken GnRH	2UXY	Aliphatic amidase	50%	100%
9	Salmon GnRH	1YY1	LHRH synthetic construct	90%	80%
10	<i>C. gariepinus</i> GnRH-R1	4GBR	Beta-2 adrenergic receptor	55%	25%
11	<i>C. gariepinus</i> GnRH-R2	4QKY	Beta-2 adrenoreceptor	76%	26%
12	Salmon GnRH-R4	4SOV	Human Ox2 orexin receptor	55%	25%

4.4.1. Homology modelling of GnRH and its receptors

GnRHs are decapeptides which bind to GnRH-R located in pituitary. Catfish GnRH (ctGnRH), chicken GnRH (cGnRH) and salmon GnRH (sGnRH) decapeptides were modelled. Ramchandran plot analysis revealed that more than 90% of the residues are in core region and none of the residues are in disallowed region for all three decapeptides (Fig. 8a-c). GnRH-Rs are G-protein coupled receptors. *C. gariepinus* GnRH-R1 (cgGnRH-R1) contains 16 alpha helices, 23 turns but no beta strand. Ramchandran plot analysis using Procheck revealed that 95% residues are in core region, 4.1% are in allowed region and 0.6% in disallowed region (Fig. 9a). GnRH-R2 of *C. gariepinus* (cgGnRH-R2) contains 14 alpha helices, 2 beta strands and 22 turns in its three dimensional structure. Ramchandran plot analysis revealed that 93.2% of the total residues are in core region, 6.2% in allowed region and none in disallowed region (Fig. 9b). Salmon GnRH-R (sGnRH-R4) contains 18 alpha helices, 2 beta strands and 26 turns in its 3D structure. Ramchandran plot analysis revealed that 88.9% of the total residues are in core region, 9.6% are in allowed region and 0.5% are in disallowed region (Fig. 9c). The values indicate reliability of the models.

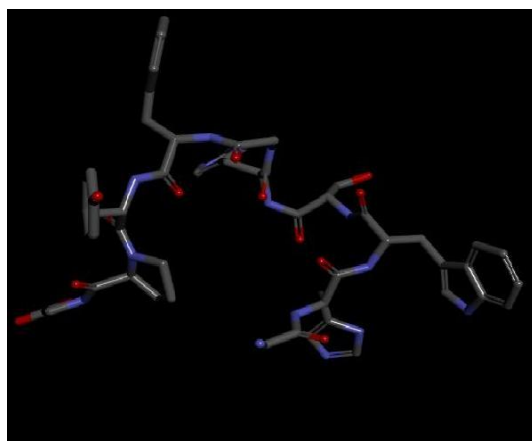
4.4.2. Homology modelling of GtH β -subunits and receptors

The 3D structures of gonadotropin hormone beta subunits are mainly composed of beta strand and turns but lack alpha helices. *C. batrachus* FSH β contains 6 beta strands and 14 turns, but no alpha helix. Procheck result for this model have revealed that 92.3% of the total residues are in the core region, 6.8% in the allowed region and only 0.9% residues are in disallowed region of the Ramchandran plot (Fig. 10a). LH β subunit of *C. batrachus* contains 8 beta strands, 15 turns and no alpha helix. Procheck result revealed that 91.8% of the total residues are in core region, 7.4% of the residues are in allowed region and none of the residues are in disallowed region of the Ramchandran plot (Fig. 10b). Three dimensional structure of *C. gariepinus* FSH β has 6 beta strands, 13 turns and no alpha helix. Ramchandran plot analysis from Procheck



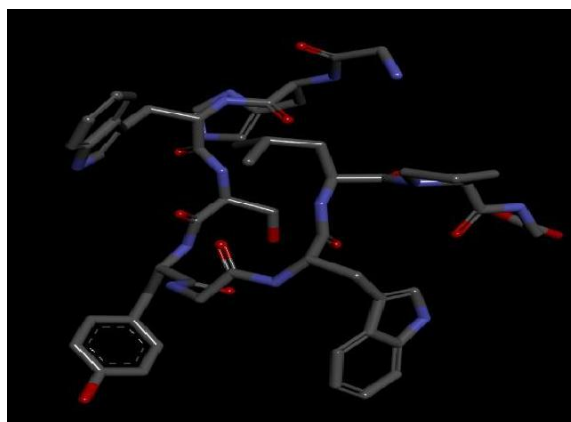
<<< P R O C H E C K S U M M A R Y >>>				
garipinus_2	2.0			10 residues
Ramachandran plot:	83.3% core	.0% allow	16.7% gener	.0% disall
Gly & Pro Ramach:	0 labelled residues (out of 2)			
Chi1-chi2 plots:	0 labelled residues (out of 6)			
Main-chain params:	6 better	0 inside	0 worse	
Side-chain params:	5 better	0 inside	0 worse	
Residue properties:	Max.deviation:	2.0	Bad contacts:	0
	Bond len/angle:	3.0	Morris et al class:	1 1 1
G-factors	Dihedrals:	.16	Covalent:	-.32
			Overall:	-.04
M/c bond lengths:	90.9% within limits	9.1% highlighted		
M/c bond angles:	90.6% within limits	9.4% highlighted		
Planar groups:	100.0% within limits	.0% highlighted		
+ May be worth investigating further. * Worth investigating further.				

a. ctGnRH



<<< P R O C H E C K S U M M A R Y >>>				
Target_1	1.3			10 residues
Ramachandran plot:	100.0% core	.0% allow	.0% gener	.0% disall
Gly & Pro Ramach:	0 labelled residues (out of 2)			
Chi1-chi2 plots:	0 labelled residues (out of 5)			
Main-chain params:	5 better	0 inside	1 worse	
Side-chain params:	5 better	0 inside	0 worse	
Residue properties:	Max.deviation:	1.4	Bad contacts:	0
	Bond len/angle:	3.4	Morris et al class:	1 2 1
1 cis-peptides				
G-factors	Dihedrals:	-.24	Covalent:	-.65
			Overall:	-.41
M/c bond lengths:	87.5% within limits	12.5% highlighted		
M/c bond angles:	79.2% within limits	20.8% highlighted		
Planar groups:	100.0% within limits	.0% highlighted		
+ May be worth investigating further. * Worth investigating further.				

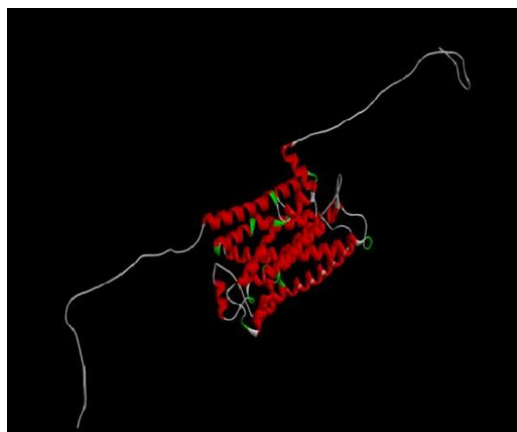
b. cGnRH



<<< P R O C H E C K S U M M A R Y >>>				
salmon_2	1.3			10 residues
Ramachandran plot:	100.0% core	.0% allow	.0% gener	.0% disall
Gly & Pro Ramach:	0 labelled residues (out of 2)			
Chi1-chi2 plots:	0 labelled residues (out of 5)			
Main-chain params:	6 better	0 inside	0 worse	
Side-chain params:	5 better	0 inside	0 worse	
Residue properties:	Max.deviation:	1.3	Bad contacts:	0
	Bond len/angle:	3.4	Morris et al class:	1 1 1
G-factors	Dihedrals:	.05	Covalent:	-.33
			Overall:	-.12
M/c bond lengths:	100.0% within limits	.0% highlighted		
M/c bond angles:	86.7% within limits	13.3% highlighted		
Planar groups:	100.0% within limits	.0% highlighted		
+ May be worth investigating further. * Worth investigating further.				

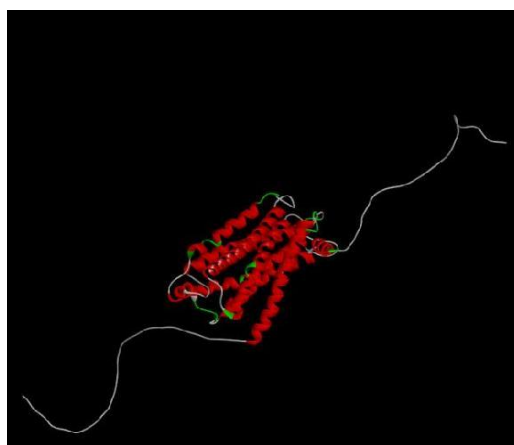
c. sGnRH

Fig. 8a, b & c. 3D structures and Procheck results of GnRH decapeptides



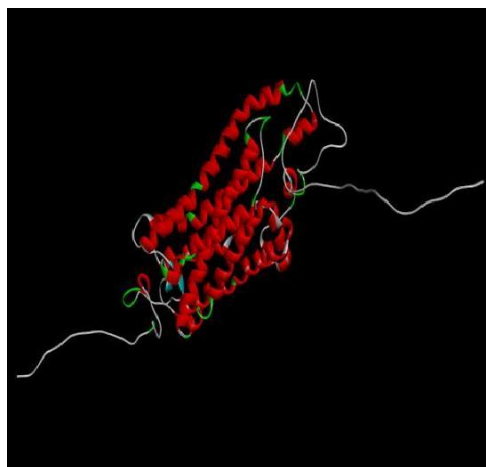
<<< P R O C H E C K S U M M A R Y >>>					
Target_1	4.0				379 residues
* Ramachandran plot:	95.8% core	4.1% allow	.3% gener	.6% disall	
+ Gly & Pro Ramach:	1 labelled residues (out of 35)				
+ Chi1-chi2 plots:	8 labelled residues (out of 216)				
Main-chain params:	6 better	0 inside	0 worse		
Side-chain params:	5 better	0 inside	0 worse		
* Residue properties:	Max.deviation:	9.2		Bad contacts:	8
	Bond len/angle:	19.3		Morris et al class:	1 1 2
G-factors	Dihedrals:	.11	Covalent:	-.33	Overall: -.05
M/c bond lengths:	87.1% within limits	12.9% highlighted			
* M/c bond angles:	72.8% within limits	27.2% highlighted			2 off graph
Planar groups:	100.0% within limits	.0% highlighted			
+ May be worth investigating further. * Worth investigating further.					

a. cgGnRH-R1



<<< P R O C H E C K S U M M A R Y >>>					
Target_1	4.0				375 residues
+ Ramachandran plot:	93.2% core	6.2% allow	.5% gener	.0% disall	
Gly & Pro Ramach:	0 labelled residues (out of 34)				
* Chi1-chi2 plots:	7 labelled residues (out of 224)				
Main-chain params:	5 better	0 inside	0 worse		
Side-chain params:	5 better	0 inside	0 worse		
* Residue properties:	Max.deviation:	17.6		Bad contacts:	8
	Bond len/angle:	5.6		Morris et al class:	1 1 2
G-factors	Dihedrals:	.12	Covalent:	-.23	Overall: -.01
M/c bond lengths:	90.6% within limits	9.4% highlighted			
M/c bond angles:	74.4% within limits	25.6% highlighted			
Planar groups:	100.0% within limits	.0% highlighted			
+ May be worth investigating further. * Worth investigating further.					

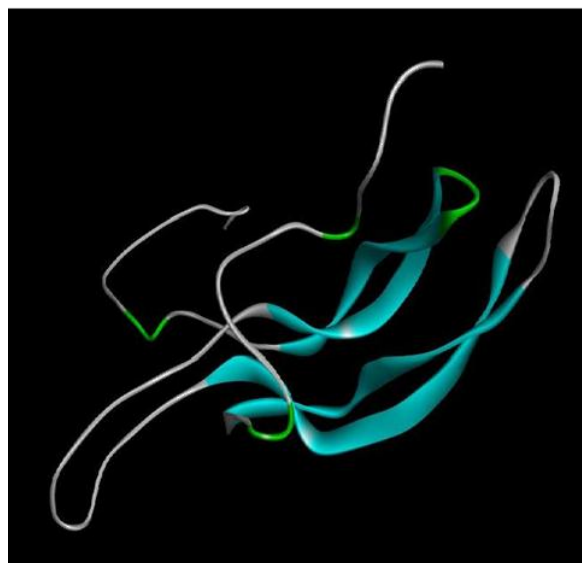
b. cgGnRH-R2



<<< P R O C H E C K S U M M A R Y >>>					
Target_2	3.7				419 residues
* Ramachandran plot:	88.9% core	9.6% allow	1.0% gener	.5% disall	
Gly & Pro Ramach:	0 labelled residues (out of 31)				
+ Chi1-chi2 plots:	4 labelled residues (out of 243)				
Main-chain params:	6 better	0 inside	0 worse		
Side-chain params:	5 better	0 inside	0 worse		
* Residue properties:	Max.deviation:	18.7		Bad contacts:	7
	Bond len/angle:	9.3		Morris et al class:	1 1 3
+ 1 cis-peptides					
G-factors	Dihedrals:	-.03	Covalent:	-.30	Overall: -.13
M/c bond lengths:	75.9% within limits	24.1% highlighted			
* M/c bond angles:	71.7% within limits	28.3% highlighted			1 off graph
Planar groups:	56.4% within limits	3.6% highlighted			
+ May be worth investigating further. * Worth investigating further.					

c. sGnRH-R4

Fig. 9a, b & c. 3D structures and Procheck results of GnRH-Rs



+-----<<< P R O C H E C K S U M M A R Y >>>-----+									
Target_9	2.9							133 residues	
* Ramachandran plot:	92.3% core	6.8% allow	.0% gener	.9% disall					
* Gly & Pro Ramach:	1 labelled residues (out of 14)								
Chi1-chi2 plots:	0 labelled residues (out of 73)								
Main-chain params:	6 better	0 inside	0 worse						
Side-chain params:	5 better	0 inside	0 worse						
* Residue properties:	Max.deviation:	8.2		Bad contacts:	2				
* Bond len/angle:	6.5		Morris et al class:	1	1	3			
G-factors	Dihedrals:	-.12	Covalent:	-.26	Overall:	-.17			
M/c bond lengths:	93.8% within limits	6.3% highlighted							
M/c bond angles:	79.1% within limits	20.9% highlighted							
Planar groups:	100.0% within limits	.0% highlighted							
+-----<<< P R O C H E C K S U M M A R Y >>>-----+									
+ May be worth investigating further. * Worth investigating further.									

a. cgFSH β



+-----<<< P R O C H E C K S U M M A R Y >>>-----+									
Target_6	2.6							138 residues	
· Ramachandran plot:	91.8% core	7.4% allow	.8% gener	.0% disall					
· Gly & Pro Ramach:	0 labelled residues (out of 14)								
· Chi1-chi2 plots:	2 labelled residues (out of 85)								
Main-chain params:	6 better	0 inside	0 worse						
Side-chain params:	5 better	0 inside	0 worse						
· Residue properties:	Max.deviation:	2.5		Bad contacts:	4				
· Bond len/angle:	5.4		Morris et al class:	1	1	3			
· 1 cis-peptides									
G-factors	Dihedrals:	-.04	Covalent:	-.33	Overall:	-.15			
M/c bond lengths:	92.9% within limits	7.1% highlighted							
M/c bond angles:	80.9% within limits	19.1% highlighted							
Planar groups:	100.0% within limits	.0% highlighted							
+-----<<< P R O C H E C K S U M M A R Y >>>-----+									
+ May be worth investigating further. * Worth investigating further.									

b. cbLH β

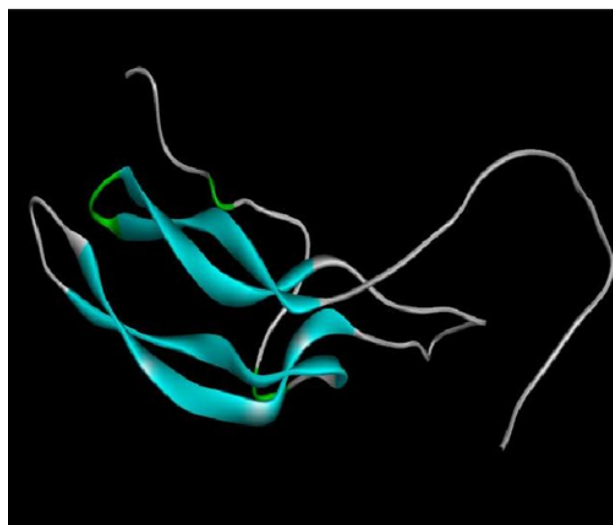
Fig. 10a & b. 3D structures and Procheck results of *C. batrachus* GtH β subunits.

revealed that 93.2% of the total residues are in core region, 5.2% in allowed region and only 0.9% in disallowed region (Fig. 11a). LH β subunit of *C. gariepinus* contains 6 beta strands, 14 turns and no helix in its three dimensional structure. Ramchandran plot analysis revealed that 87.7% of the total residues are in core region, 8.2% in allowed region and none of the residues are in disallowed region hence quality of model is good as there is no residue in disallowed region (Fig. 11b). All the models are of good quality.

All gonadotropin hormone receptors are seven transmembrane rhodopsin family receptors, which are G- protein coupled receptors. The ectodomains of the receptors that are important for the hormonal binding have been modelled. The ectodomain of FSH receptor of *C. gariepinus* contains 1 alpha helix, 58 beta-strands and 52 turns. Ramchandran plot analysis by Procheck revealed that 86.6% of the total residues are in core region, 12.5% in the allowed region and 0.7% in disallowed region (Fig. 12a). LH-R of *C. gariepinus* contains 1 alpha helix, 41 beta strands and 50 turns in its 3D structure. Ramchandran plot analysis revealed that 87.5% of the total residues are in core region, 11.0% in the allowed region and 0.3% residues in disallowed region (Fig. 12b).

4.4.3. Docking between hormones and receptors

Docking studies between reproductive hormones with their receptors were carried out. Rigid body docking, exclusively based on shape complementarity criteria, was performed first. Then the results were refined by using flexible docking tool. Electrostatic bonds, hydrogen bonds and hydrophobic interactions were observed. Differential affinities were evaluated based on number of interactions.



<<< P R O C H E C K S U M M A R Y >>>				
Target_10	2.9			133 residues
* Ramachandran plot:	93.2% core	5.1% allow	.9% gener	.9% disall
Gly & Pro Ramach:	0 labelled residues (out of 14)			
Chi1-chi2 plots:	0 labelled residues (out of 74)			
Main-chain params:	6 better	0 inside	0 worse	
Side-chain params:	5 better	0 inside	0 worse	
* Residue properties:	Max.deviation:	4.0	Bad contacts:	0
* Bond len/angle:	6.5	Morris et al class:	1	1 3
G-factors	Dihedrals:	-.11	Covalent:	-.29 Overall: -.17
M/c bond lengths:	93.6% within limits	6.4% highlighted		
M/c bond angles:	78.9% within limits	21.1% highlighted		
Planar groups:	100.0% within limits	.0% highlighted		
+ May be worth investigating further. * Worth investigating further.				

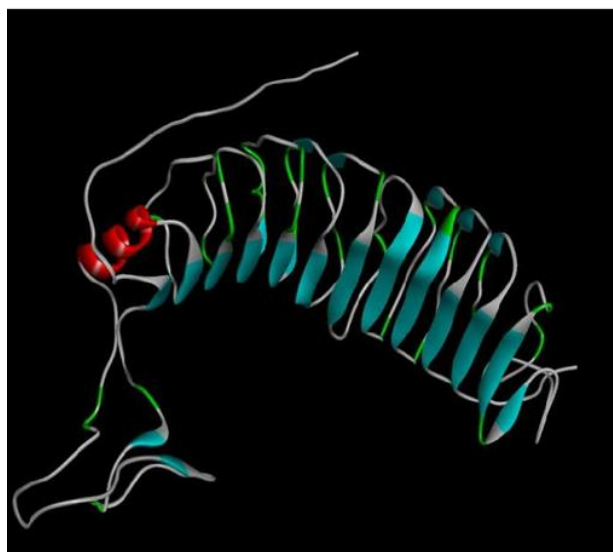
a. cgFSH β



<<< P R O C H E C K S U M M A R Y >>>				
Target_3	2.6			138 residues
+ Ramachandran plot:	87.7% core	8.2% allow	4.1% gener	.0% disall
Gly & Pro Ramach:	0 labelled residues (out of 14)			
+ Chi1-chi2 plots:	1 labelled residues (out of 84)			
Main-chain params:	6 better	0 inside	0 worse	
Side-chain params:	5 better	0 inside	0 worse	
* Residue properties:	Max.deviation:	3.5	Bad contacts:	3
* Bond len/angle:	6.2	Morris et al class:	1	1 3
+ 1 cis-peptides				
G-factors	Dihedrals:	-.12	Covalent:	-.39 Overall: -.22
M/c bond lengths:	91.4% within limits	8.6% highlighted		
M/c bond angles:	76.9% within limits	23.1% highlighted		
Planar groups:	100.0% within limits	.0% highlighted		
+ May be worth investigating further. * Worth investigating further.				

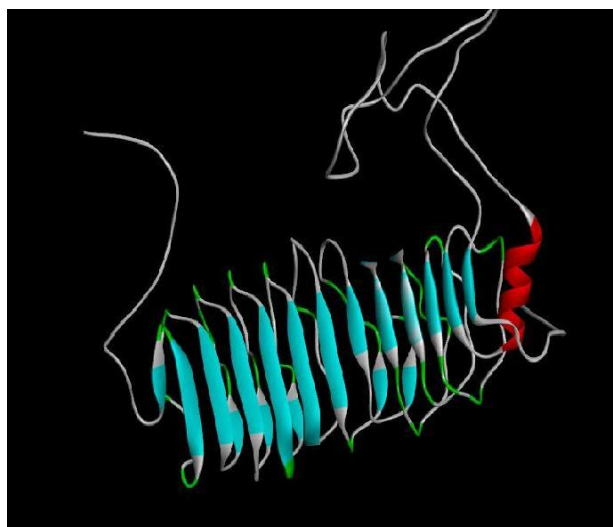
b. cgLH β

Fig. 11a & b. 3D structures and Procheck results of *C. gariepinus* GtH β subunits



-----<<< P R O C H E C K S U M M A R Y >>>-----					
Target_5	2.5				662 residues
Ramachandran plot:	86.6% core	12.5% allow	.2% gener	.7% disall	
Gly & Pro Ramach:	0 labelled residues (out of 54)				
Chi1-chi2 plots:	15 labelled residues (out of 398)				
Main-chain params:	6 better	0 inside	0 worse		
Side-chain params:	5 better	0 inside	0 worse		
Residue properties:	Max.deviation:	4.0		Bad contacts:	6
	Bond len/angle:	6.4		Morris et al class:	1 1 3
2 cis-peptides					
G-factors	Dihedrals:	-.11	Covalent:	-.28	Overall: -.17
M/c bond lengths:	84.8% within limits	15.2% highlighted			
M/c bond angles:	74.7% within limits	25.3% highlighted			
Planar groups:	100.0% within limits	.0% highlighted			
+ May be worth investigating further. * Worth investigating further.					

a. cgFSH-R



-----<<< P R O C H E C K S U M M A R Y >>>-----					
Target_6	2.5				710 residues
Ramachandran plot:	87.5% core	11.0% allow	1.2% gener	.3% disall	
Gly & Pro Ramach:	2 labelled residues (out of 60)				
Chi1-chi2 plots:	21 labelled residues (out of 421)				
Main-chain params:	6 better	0 inside	0 worse		
Side-chain params:	5 better	0 inside	0 worse		
Residue properties:	Max.deviation:	4.8		Bad contacts:	6
	Bond len/angle:	8.3		Morris et al class:	1 1 3
2 cis-peptides					
G-factors	Dihedrals:	-.09	Covalent:	-.29	Overall: -.16
M/c bond lengths:	84.9% within limits	15.1% highlighted			
M/c bond angles:	73.3% within limits	26.7% highlighted		1 off graph	
Planar groups:	100.0% within limits	.0% highlighted			
+ May be worth investigating further. * Worth investigating further.					

b. cgLH-R

Fig. 12a & b. 3D structures and Procheck results of *C. gariepinus* GtH receptors

4.4.3.1. GnRH docking with receptors

The interaction of catfish and salmon GnRH receptors with three GnRH variants, namely catfish GnRH (ctGnRH), chicken GnRH (cGnRH) and salmon GnRH (sGnRH) have been studied.

GnRH-R1 of *Clarias gariepinus* (cgGnRH-R1) does not seem to be interacting with catfish GnRH (ctGnRH) as no bonds are predicted after docking. But this receptor interacts with chicken and salmon GnRH (Table 3a). However, the interaction between cgGnRH-R2 and ctGnRH is strongest with 5 electrostatic, 8 hydrogen and 5 hydrophobic bonds. On the other hand, the interaction of cGnRH and sGnRH is stronger with cgGnRH-R1 compared with cgGnRH-R2. Surprisingly, the salmon GnRH-R seems to interact more strongly with the ctGnRH than its homologous ligand. A qualitative estimation of strength of binding on the basis of number electrostatic and H-bonds has been shown in Table 3b.

4.4.3.2. GtH β -subunits docking with receptors

Docking studies of *C. batrachus* and *C. gariepinus* FSH β and LH β with homologous and heterologous receptors revealed that unlike previous reports, CgLH-R can interact with both LH β and FSH β , showing promiscuous binding (Table 4a). Moreover, the *C. batrachus* LH β interacts more strongly with CgLH-R compared with its homologous ligand. Another significant observation was that CgFSH-R interacts strongly with LH β , indicating that neither the catfish hormones nor their receptors have completely evolved into their specific roles observed in mammals. A qualitative estimation of strength of binding on the basis of number electrostatic and H-bonds has been shown in Table 4b.

Table 3a. Interaction between GnRH with its receptors

Complex	Interaction
cgGnRH-R1 - catfish GnRH	No interaction
cgGnRh-R1 – chicken GnRH	2 electrostatic + 6 hydrogen
cgGnRH-R1 – salmon GnRH	1 hydrophobic
cgGnRh-R2 – catfish GnRH	5 electrostatic + 8 hydrogen + 5 hydrophobic
cgGnRH-R2 – chicken GnRH	1 electrostatic + 5 hydrogen + 5 hydrophobic + 1 other
cgGnRH-R2 – salmon GnRH	2 hydrogen + 5 hydrophobic
sGnRH-R - catfish GnRH	2 electrostatic + 6 hydrogen + 2 hydrophobic
sGnRH-R - chicken GnRH	1 electrostatic + 1 hydrophobic
sGnRH-R – salmon GnRH	1 electrostatic + 3 hydrogen + 4 hydrophobic

Table 3b. Qualitative ranking of interactions

Receptor \ Hormone	ctGnRH	cGnRH	sGnRH
cgGnRH-R1	No interaction	Moderate	Moderate
cgGnRH-R2	Very strong	Strong	Moderate
sGnRH-R4	Very strong	Moderate	Strong

Table 4a. Interaction of gonadotropins with receptors

Complex	Type of interaction
FSH-R - cbFSH	5 – Hydrogen bonds
FSH-R - cgFSH	4 – Hydrogen bonds + 1 hydrophobic
FSH-R – cbLH	3 – Electrostatic + 7 hydrogen + 6 hydrophobic + 1 others
FSH-R – cgLH	4 – Electrostatic + 3 hydrogen + 2 hydrophobic
LH-R – cbFSH	2 – electrostatic + 6 hydrophobic + 2 others
LH-R – cgFSH	1 Electrostatic + 9 hydrogen + 3 hydrophobic + 1 others
LH-R – cbLH	6 Electrostatic + 17 hydrogen bond + 29 hydrophobic + 2 Others
LH-R – cgLH	1 Electrostatic + 1 hydrophobic

Table 4b. Qualitative ranking of interactions

Receptor \ Hormone	cbFSH β	cgFSH β	cbLH β	cgLH β
cgFSH-R	Moderate	Moderate	Strong	Strong
cgLH-R	Strong	Strong	Very strong	Moderate

5. DISCUSSION

Production of good quality seed of the commercially important catfishes including *Clarias batrachus*, the Indian magur, is a challenge for aquaculturists. A number of fishes can be induced to mature and breed in captivity either by manipulating external environmental parameters like photoperiod, water temperature and spawning substrate or by giving exogenous hormonal formulations. In fact, exogenous hormone administration has been very successfully used in several fish species to induce spermiation in males and ovulation in females. Hormonal manipulations for cultured fishes mainly involve the use of gonadotropins, mainly LH preparations, that directly stimulate the gonad, or synthetic analogues of gonadotropin-releasing hormone (GnRHa) that induce the pituitary to release endogenous gonadotropins (Mylonas *et al.*, 2010). Unfortunately, this strategy, though successful in other catfish and even in the magur female, does not work very efficiently in the male that has to be sacrificed for collection of sperms. Moreover, due to habitat destruction and introduction of the fast growing exotic species, *Clarias gariepinus*, natural stocks of this fish are dwindling (Hossain *et al.*, 2006; Ahmed *et al.*, 2012) and it is classified as endangered. Even though it is a high priced fish its culture is not popular due to paucity of seed. A better understanding of the magur reproductive endocrinology is very crucial to achieve a breakthrough in this area. Up till recently, very little molecular information was available on this species and hence, application of bioinformatics to specific hormone-receptor interactions was not possible. Sequences of *C. batrachus* GnRH genes that play a vital role in reproduction, GTH-II β , P450_{arom} and Vasa coding regions have been recently reported from this lab.

In teleosts, two types of gonadotropins were first identified in chum salmon, *Oncorhynchus keta* (Kawauchi *et al.*, 1986), and named gonadotropin I and II (GtH-I and GtH-II). GTH-I and GTH-II are now generally accepted as homologues of mammalian FSH and LH, respectively. The alpha subunit of these two proteins

(GtHα) is common and has been sequenced for the first time from *C. batrachus* in this work. Totally 475 bp of this transcript could be amplified, while the 5' terminus could not be completed. The missing region, as seen from the nearest *C. gariepinus* ortholog (91% identity) is ~190 bp. The sequence had significant similarity with other catfishes like *Heteropneustes fossilis* (90%) and *Ictalurus punctatus* (82%). These values show the highly conserved nature of this gene which is in accordance with the findings in other teleosts (reviewed by Levavi-Sivan *et al.*, 2010).

Based on observations made in *C. gariepinus* (Vischer *et al.*, 2002), the FSHβ subunit (also sequenced in this work) is believed to act at early stages of testicular development to regulate testicular function in *C. batrachus*. The 669 bp sequence that could be generated here lacks ~130 bp of the 5'-terminus, an estimation based on the closest *C. gariepinus* ortholog (93%). Once again the sequence seems to be highly conserved among catfishes, showing 92% and 82% homologies with *H. fossilis* and *I. punctatus*, respectively.

Fish LH-R like mammalian LH-R is a G-protein-coupled receptor containing a large extracellular domain followed by seven transmembrane helices and a carboxy-terminal intracellular tail. Cloning and functional characterization of LH-R in African catfish has been done earlier (Vischer & Bogerd, 2003). Only 1064 bp of the *C. batrachus* LH-R gene could be sequenced here, with information of ~430 and ~880 bp missing from 5' and 3' termini, respectively. The generated sequence showed highest identity with *I. punctatus* LH-R.

GnRH-R belongs to the G-protein coupled receptor family, which contain an N-terminal extracellular domain (30-40 aa), a larger transmembrane domain (280-290 aa), and a short C-terminal tail. Presence of C-terminal tail is characteristic of fish GnRH-R, but it is absent in mammalian GnRH-R. Two GnRH-R variants have reported in African catfish, cfGnRH-R1 and cfGnRH-R2, and both have greater affinity for chicken GnRH II compared to the homologous cfGnRH. However, they differ in tissue distribution (Lethimonier *et al.*, 2003). Totally 342 bp of this transcript could be amplified from *C. batrachus* in this study, and the entire sequence is internal.

The ligand selectivity of mammalian GtHRs is well defined and FSH-R and LH-R bind their respective ligands specifically with little cross-activation (0.01-0.1%) even in presence of physiologically relevant hormone concentrations (reviewed by Levavi-Sivan *et al.*, 2010). In contrast, the binding specificities are less well differentiated in fishes, resulting in promiscuous hormone-receptor interactions. Earlier workers (Kwok *et al.*, 2005) used radiolabelled hormones to demonstrate promiscuous interactions between hormones and receptors in coho salmon. It was reported that though FSH-R responds to both FSH and LH, LH-R binds only to LH. In case of African catfish and zebrafish, the studies carried out in transfected cell lines were based on rise in cAMP levels due to receptor activation. In both species, FSH-R responds to both FSH and LH but LH-R respond only to LH (reviewed by Levavi-Sivan *et al.*, 2010). In present work, homology modelling and docking studies indicate that both cgFSH-R and cgLH-R respond to both gonadotropins showing lack of specificity. In addition, higher interaction of cgFSH-R is predicted with the heterologous cbLH β compared to the homologous ligand. It is not always necessary that homologous ligands have the highest interaction, as seen earlier in the case of cfGnRH receptors that show affinity to chicken GnRH (reviewed by Lethimonier *et al.*, 2004). The *in silico* predictions made here will need to be further tested biochemically.

In case of GnRH receptors, a number of variants have been reported from a single species and ligand selectivity studies have also been done in some teleosts. There are two GnRH-R variants in goldfish, GfA and GfB, both showing similar binding preferences, with affinities decreasing in the order of chicken GnRH > salmon GnRH > mammalian GnRH > species specific GnRH. Once again, the homologous ligand shows less affinity. African catfish codes for two GnRH-R variants and binding studies revealed the ligand selectivity order for GnRH-R1 as cGnRH II > sGnRH = mGnRH = ssGnRH, while GnRH-R2 bound with sGnRH > mGnRH > ssGnRH in a decreasing order of specificity. In striped bass there is only one receptor, for which the order of preference is cGnRH > mGnRH = sGnRH > ssGnRH (reviewed by Lethimonier *et al.*, 2004). In our study, cgGnRH-R2 seems to respond with more strength to ctGnRH compared with sGnRH and cGnRH, this time showing preference for the homologous sequence. Here we have predicted the order of selectivity for

cgGnRH-R1 as cGnRH > sGnRH and for cgGnRH-R2 as ctGnRH-R > cGnRH > sGnRH.

Induced maturation of fish in captivity is currently being performed by potent synthetic GnRH analogues (GnRHa). These GnRH analogues viz. mammalian GnRHa, and salmon GnRHa contain D- amino acid in place of natural one at 6th position and ethylamide at the place of 10th amino acid. These modifications give them resistance against enzymatic cleavage (Zohar *et al.*, 2001). The use of sGnRHa is very common in aquaculture, but as shown in the present work, both GnRH-R variants of *C. gariepinus* do not interact strongly with sGnRH. In fact, cgGnRH-R2 interacts more strongly to ctGnRH. Although, the *C. batrachus* GnRH receptors could not be completely sequenced in this work, observations from the very closely related *C. gariepinus* sequence, indicate that synthesis of a species specific GnRH analogue might yield better results in catfish induced breeding.

6. SUMMARY

Aquaculture is one of the fastest growing food industries and aquatic animals are an important source of protein rich food throughout the globe (Ayyappan *et al.*, 2006). In India, over 80% of the total inland fish production comprises of carps. Though catfishes fetch a good price in the domestic fish market due to good taste and higher nutritional value, their aquaculture is not widespread. *Clarias batrachus* commonly referred to as the Indian magur, is a native catfish of India, whose culture is restricted due to limited seed availability. Both males and females mature in captivity and on induction by gonadotropin releasing hormone (GnRH) females release eggs on stripping. Males, however, do not readily ooze milt on stripping and need to be either sacrificed or surgically operated to obtain testis that are macerated to obtain sperms. This problem also obstructs the implementation of suitable genetic selection program and the fast growing exotic *Clarias gariepinus* is preferred over the indigenous species. Use of synthetic inducers is presently a common aquaculture practice that is indispensable in most cases. However, all fishes do not respond in a similar manner because of their specific endocrine regulatory mechanisms. More potent biomolecules are required for species like magur and this can be done by implementing the concepts of structural biology to identify and design new molecules targeted to specific needs. Full length coding sequences of *C. batrachus* GnRH, luteinizing hormone β subunit (LH β), cytochrome 450 aromatase and vasa genes known to play major roles in reproductive physiology, were generated in our lab earlier. However, information is not available on receptors GnRH-R and LH β -R. In addition, *C. batrachus* follicle stimulating hormone β subunit (FSH β) and the gonadotropin α subunit (GtH α) have also not been sequenced. In this context, the present study was planned with the following objectives.

- Amplification and sequencing of gonadotropin releasing hormone receptor, gonadotropin hormone and receptor genes of *Clarias batrachus*.
- Homology modelling and docking studies on reproductive hormones and receptors of *Clarias batrachus*.

Degenerate primers were designed to PCR amplify partial sequences of GnRH-R, LH β -R, FSH β and GtH α from cDNA prepared from brain and testis tissue of mature male *C. batrachus*. Alignment of sequences from closely related species was done by Clustal W and GeneRunner v 3.0 software was used for primer designing. Using standard genetic engineering protocols (Sambrook *et al.*, 2001), the amplicons were excised from agarose gels and TA cloned into pTZR/T vector (Fermentas, USA) and commercially sequenced (Xcelris Genomics Ltd., India). 327, 1036, 213 and 201 bp of GnRH-R, LH β -R, FSH β and GtH α genes were obtained, respectively. To determine sequences of 3' & 5' termini, rapid amplification of cloned ends was done using RACE kit (Life Technologies, USA). Only GtH α and FSH β sequences could be extended by 274 and 456 bp, respectively, on the 3' ends. 5' ends could not be obtained for any partial sequence.

Homology modelling and docking studies were done to determine binding efficiencies of *C. batrachus* FSH β and LH β with corresponding receptors from *C. gariepinus* and it was found that *C. batrachus* LH β binds more efficiently to the heterologous LH-R compared to the homologous pair. All other bindings were similar. A significant observation was made when binding of salmon, chicken and catfish GnRH was tested with salmon receptor and catfish (*C. gariepinus*) GnRH-R1 and R2. The *C. batrachus* GnRH showed much stronger interaction with catfish GnRH-R2 and only moderate binding to salmon receptor. More importantly, the interaction of salmon GnRH with catfish GnRH receptors was strong.

The present work provides important insight into the reasons why the commercial formulations of GnRH currently in use are not very effective for catfish, especially magur. However, results obtained from *in silico* studies need to be validated *in vivo* and induced breeding with catfish specific GnRH may be tested in future studies.

7. REFERENCES

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ABBREVIATIONS

µg	Microgram (10^{-6} g)
µl	Microlitre (10^{-6} l)
µM	Micro molar
°C	Degree centigrade
Amp	Ampicillin
BLAST	Basic Local Alignment Search Tool
Bp	Base pair
cDNA	Complementary DNA
Conc.	Concentration
DEPC	Diethyl Pyro Carbonate
DMW	Demineralized water
DMSO	Dimethyl Sulphoxide
DW	Distilled water
DNA	Deoxyribonucleic acid
DNAase	Deoxyribonuclease
dNTP	Doxynucleotide triphosphate
EDTA	Ethylene diamine tetra acetic acid
Fig.	Figure
G	Gram
H	Hour(s)
IPTG	Isopropyl-thiogalactopyranoside
Kg	Kilogram
L	Litre

LB	Luria-bertani
M	Molar
mg	Milligram (10^{-3} g)
Min	Minute
ml	Milliliter (10^{-3} L)
mM	Milli molar
Mol	Mole
mRNA	Messenger RNA
MW	Molecular weight
N	Normal
ng	Nano gram
nm	Nanometer
PCR	Polymerase chain reaction
Pmol	Pico moles
ppm	Parts per million
Ppt	Parts per thousand
RE	Restriction Enzyme
RNA	Ribonucleic Acid
RNAase	Ribonuclease
rpm	Revolution per minute
rRNA	Ribosomal RNA
r.t.	Room temperature
s	Second
Sec.	Section
TAE	Tris acetate EDTA
TE	Tris-EDTA buffer
U	Unit

V	Volt
Vol	Volume
X-gal	5-bromo-4-chloro-3-indolyl-b galactopyranoside
DO	Dissolved Oxygen
USD	U. S. Dollar
pH	Potentio Hydrogeny
UV	Ultra Violet
Rpm	Revolution per minute
GtH α	Gonadotropin alpha subunit
FSH β	Follicle stimulating hormone beta subunit
LH-R	Luteinizing hormone receptor
GnRH-R	Gonadotropin-releasing hormone receptor

ANNEXURE I

Reagent Composition

1. Bacterial strains and culture

1.1. LB -Agar medium (Luria Bertani Agar)

Tryptone	2g
NaCl	1g
Yeast extracts	3g
Agar	2g
DMW	200 ml

Sterilized by autoclaving.

1.2. Ampicillin (100 mg/ml)

Ampicillin sodium salt	100 mg
DMW	600 μ l
Ethyl alcohol	400 μ l

(Used in the media at 100 μ g/ml concentration)

2. Plasmid DNA Isolation

2.1. PI Lysis buffer

Glucose	5 ml of 1 M glucose
Tris-Cl (pH 8.0)	2.5 ml of 1M Tris-Cl
EDTA (pH 8.0)	2 ml of 0.5 M EDTA

Make the volume to 100 ml and sterilized by autoclaving, stored at 4°C.

2.2. PII Lysis buffer

NaOH	0.2N (Freshly diluted from 10N stock)
SDS	1%

2.3. PIII Lysis buffer

5M Potassium acetate (See below)	60.0 ml
Glacial acetic acid	11.5 ml
DMW	28.5 ml
Stored at 4°C.	

2.4. Potassium acetate (5M)

Potassium acetate	49.10 g
DMW	100 ml
pH adjusted to 7.5 by 2M acetic acid and sterilized by autoclaving.	

2.5. Diethylpyrocarbonate (DEPC) Water

DEPC	1ml
Sterile dH ₂ O	1 liter

Allowed to stand for 1hr at 37°C or overnight at room temperature and then autoclaved for 15 min at 15 psi (1.05 kg/cm²).

3. S.N.A.P. Column Purification

3.1. 1X Wash Buffer for S.N.A.P. Procedure

Wash buffer concentrate	1 ml
D/W	18 ml
Absolute ethanol	21 ml
Transfer to an appropriate-sized glass bottle. Cap and store at 4°C.	

3.2. 70% Ethanol Wash for S.N.A.P. Procedure

Absolute ethanol	35 ml
D/W	15 ml
Transfer to an appropriate-sized glass bottle. Cap and store at 4°C.	

3. 2.Preparation of recombinant DNA constructs

EDTA	18.61 g
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DMW	80 ml
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pH adjusted to 8.0 with NaOH. Make up the volume to 100 ml and sterilize by autoclaving.

3.2. 1 M Glucose

Glucose	180.16 g
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Dissolve in 1000 ml of distilled water.

3.3. Tris-Cl (1 M; pH 8.0)

Tris base	12.11 g
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DMW	200 ml
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pH adjusted to 8.0 with conc. HCl and sterilized by autoclaving.

3.4. TAE (50X)

Tris base	242 g
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Glacial acetic acid	57.1 ml
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EDTA (0.5 M pH 8.0)	100 ml
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Final volume made to 1000 ml.

3.5. Ethidium Bromide (10 mg/ml)

Ethidium bromide	0.1 g
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DMW	10 ml
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Covered with aluminium foil and stored at 4°C.

3.6. Gel loading buffer

Bromophenol blue	0.25 g
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Xylene cyanol	0.25 g
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Glycerol	3 ml
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3.7. Calcium chloride (0.1 M)

CaCl ₂ .6H ₂ O	2.2g
DMW	100ml

Sterilized by filtration through a 0.22 micron disposable filter; Stored in 1ml aliquots at 4°C.

3.8. IPTG [Isopropyl thio-b-D- galactoside (MW 283.3)]

IPTG	2 g
DMW	10 ml

Sterilized by filtration through a 0.22 micron disposable filter; Stored at -20°C.

3.9. X-gal (2% w/v)

Xgal	20 mg
Dimethyl formamide	1 ml

The stock solution is made at the concentration of 20 mg/ml and Stored at -20°C.

