DEVELOPMENT AND NANO-DELIVERY OF CHITOSAN CONJUGATED STEROIDOGENIC ACUTE REGULATORY PROTEIN (StAR) GENE CONSTRUCT IN *CLARIAS BATRACHUS*

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

M.F.Sc. (Fish Genetics and Breeding)

By

PRAVESH KUMAR, B.F.Sc
(FGB- MA3- 03)

ICAR-CENTRAL INSTITUTE OF FISHERIES EDUCATION
(University Established under Sec. 3 of UGC Act 1956)
Panch Marg, off Yari Road, Versova, Andheri (W),
Mumbai–400 061

JUNE 2015

Kumar, P., 2015. Development and nano-delivery of chitosan conjugated steroidogenic acute regulatory protein (StAR) gene construct in *Clarias batrachus*. Central Institute of Fisheries Education (University Established under Sec. 3 of UGC Act 1956), Panch Marg, Off Yari Road, Versova, Andheri (W), Mumbai–400 061.

Dedicated

To

My parents, Teachers

And Friends

Date: 30th June, 2015

CERTIFICATE

Certified that the dissertation entitled **"DEVELOPMENT AND NANO-DELIVERY OF CHITOSAN CONJUGATED STEROIDOGENIC ACUTE REGULATORY PROTEIN (StAR) GENE CONSTRUCT IN *CLARIAS BATRACHUS*"** is a record of independent bonafide research work carried out by **Mr. Pravesh Kumar** during the period of study from September 2014 to June 2015 under our supervision and guidance for the degree of **Master of Fisheries Science (Fish Genetics and Breeding)** and that the dissertation has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or any other similar title.

Major Advisor/Chairman

(Rupam Sharma)
Senior Scientist
Fish Genetics and Biotechnology
Division

Advisory Committee

(Gireesh Babu P.)
Scientist
Fish Genetics and Biotechnology
Division

(Kundan Kumar)
Scientist
Aquatic Animal Health
Management Division

(Suresh Babu P.P.)
Scientist
CMFRI, Kochi

DECLARATION

I hereby declare that the dissertation entitled "**DEVELOPMENT AND NANO-DELIVERY OF CHITOSAN CONJUGATED STEROIDOGENIC ACUTE REGULATORY PROTEIN (StAR) GENE CONSTRUCT IN *CLARIAS BATRACHUS***" 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.

30th June 2015
Mumbai

(Pravesh Kumar)
M.F.Sc.Student
Fish Genetics and Breeding Division, CIFE

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.

First of all, I would like to convey my heartfelt gratitude and respect to my supervisor ***Dr. Rupam Sharma,*** *senior scientist who helped me throughout my dissertation work. Without 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 love.*

I would like to express my gratitude and thanks to my advisory committee member ***Dr. Gireesh Babu P****. sir for his constant support, useful suggestions and kind help during the entire period of my research work.*

I express my gratitude ***to Dr. Kundan Kumar*** *sir and* ***Dr. Suresh Babu P.P.*** *sir who as member of the advisory committee always offered wholehearted support and guidance during this research work.*

I am also thankfull to ***Dr. Aparna Choudhari*** *m'aam,* ***Dr. S. Jahagirdar*** *Sir,* ***Dr. A. Pavan Kumar*** *Sir,* ***Dr. Makesh*** *sir****, Dr. C. S. Chaturvedi*** *Sir and* ***Mujahid*** *sir for their kind support and help whenever needed throughout my research work.*

It is my honor and great privilege to express my deep sense of gratitude to ***Dr. W.S. Lakra****, Director/Vice-Chancellor, CIFE,* ***Dr. A. K. Pal*** *(Ex-Joint Director),* ***Dr. N.P. Sahoo****, Dean (Academics), CIFE and* ***Dr. Gopal Krishna****, HOD, FGB Division for providing the necessary facilities needed for conducting the research.*

I want to express my extreme thanks to ***Dr. Arun Sharma****, Scientist-In-charge, Kakinada centre, CIFE,* ***Dr. Ibemcha Chanu*** *ma'am and* ***Dr. Srinivas Rav*** *for their kind help during my experiment setup and sample collection.*

I am bound and privileged to mention the name of ***Asraf*** *Sir,* ***Gajanan*** *sir,* ***Dhaval*** *sir and* ***Sujit*** *sir for their support, love, care and guidance during the entire research work. To them I offer my heartiest gratitude and thankfulness.*

I am in debt to my beloved roommate ***Anupam*** *for his help, support, care and love which will always remain printed in my heart, my seniors* ***Irfan, Partha, Ratandeep, Aditya, Deepak, Rajan, Jaffer, Adnan, Mukesh, Supriya*** *Ma'am,* ***Niti*** *Ma'am,* ***Nilambari*** *Ma'am,* ***Ishita*** *Ma'am and* ***Yogita*** *Ma'am for their suggestions, everlasting encouragement, care and constant support throughout my research period.*

I shall ever remain indebted to my classmates. I have no words to express my sincere thanks to for their immense help at each and every step of my dissertation work. The affection, assistance and encouragement provided by my classmates ***Amol, Manu, Sahana, Mrinmoyee, Nishant, Gulsan, Shudhansu, Nilav, Priynka*** *and* ***Ganesh*** *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,* ***Resma*** *m'aam,* ***Sanjay*** *bhai,* ***Patil*** *bhai,* ***Jena*** *bhai* ***Jetu*** *bhai,* ***Biswas*** *ji, and* ***kavita*** *who had all bestowed their valuable time in cooperation, support, and helped me throughout my research period.*

I want to thank whole heartedly to all my friends ***Karan, Naresh, Pram, Harendra, Anil, Aashish, Irasad and Shyamlal,*** *who are far and near to me, but always by my side when ever needed and guide me all the way mentally, spiritually and financially.*

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 am finding short of words to express my wholehearted love, boundless gratitude to my beloved parents, my sister and all family members whose selfless, uninterrupted love, empathy, sympathy, blessing, encouragement, sacrifice and support are the constant source of my inspiration and whose guidance always lead me towards the right path. Without them I would not have reached where I am now.

I am grateful to the librarian and all staff members of ***CIFE Library*** *for their substantial assistance and cooperation to complete all the relevant literature.*

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

Date: 30/06/15 **(PRAVESH KUMAR)**

सारांश

एशियाई केटफिस, क्लेरियस बेटेरेकस को लोकप्रिय रूप से मांगुर कहा जाता हैं। अच्छे स्वाद, उच्च पोषण और बाजार मे जादा मांग के कारण यह एक व्यावसायिक रूप से महत्वपूर्ण प्रजाति है। लेकिन क्रत्रिम प्रजनन मे समस्या की वजह से मांगुर के बच्चे जल उच्छेर के लिये उपलब्ध नही हो पा रहे हे। प्रजनन मे समस्या प्रमुख रूप से नर मछली से संबन्धित हे जो हॉर्मोनल इंजेक्सेन के बाद भी मिल्ट नही निकाल पाती हे ओर इसीलिए हर बार नर को मारना पडता हे। वर्तमान अध्धेयन स्टार जीन पर केन्द्रित हे जिसकी स्टेरॉइडोजनेसिस मे एक महत्वपूर्ण भूमिका है। स्टार जीन को क्लेरियस बेटेरेकस के वृषण से प्रवर्धित करके एक्स्प्रेसन वेक्टर pcDNA4/HisMax A मे क्लोन किया गया। स्टार जीन कंस्ट्रक्ट (pcDNA4-StARcds) की कार्यक्षमता एसएसएन-1 सेल लाइन में ट्रांसफेक्ट करके पुस्ट की गई। एसडीएस पेज एवम वेस्टर्न ब्लोटिंग विश्लेषण से पता चला की एक 30 kDa पुनः संयोजक प्रोटीन कोशिकाओं के अंदर बन रही हे। कंस्ट्रक्ट को 135.4 नैनो मीटर व्यास और 17.7 एम वी जीटा पोटेन्सियल के काइटोसन नैनोकणों के साथ संयुग्मित किया गया। काइटोसन नैनोकणों के साथ कंस्ट्रक्ट की कॉम्प्लेक्स बनाने की छमता एवम endonucleases के क्षरण के खिलाफ स्थिरता एक जटिल प्रपत्र जेल मंदता परख द्वारा पुष्टि की गई। इंट्रामस्क्युलर इंजेक्सेन के 5 वें दिन कंस्ट्रक्ट वृषण, मस्तिष्क, सिर गुर्दे और मांसपेशियों से एंप्लीफाइड किया जा सकता है। चयनित प्रजनन जीन CYP11A1, CYP17A1, 3-β-एचएसडी, 17-β-एचएसडी और P450 एरोमाटेज़ मे नियंत्रण की तुलना में नैनो संयुग्मित प्रशासित समूह में सभी चयनित जीनों की अभिव्यक्ति के स्तर में उल्लेखनीय वृद्धि देखी गई है, जो वास्तविक समय पीसीआर द्वारा विश्लेषण किया गया था। नेकड कंस्ट्रक्ट ग्रुप की मछलियो की अभिव्यक्ति में कोई उल्लेखनीय बदलाव नहीं आया था। 11-के.टी. एवम एस्ट्राडियोल हार्मोन में वृद्धि और वृषण ऊतक की संरचना में परिवर्तन जेसे सेमिनीफेरस नलिकाओं में प्राथमिक शुक्राडु कोशिकाओं ओर शुक्राणुओ मे वृद्धि नैनो संयुग्मित काइटोसन के संयुग्मित निर्माण की प्रभावकारिता और प्रजनन करने में उसकी भूमिका बताता है। वर्तमान अध्ययन क्लेरियस बेटेरेकस में काइटोसन संयुग्मित स्टार जीन कंस्ट्रक्ट के वितरण पर पहली रिपोर्ट है।

ABSTRACT

The Asian catfish, *Clarias batrachus* popularly known as magur is a commercially important species due to its good taste, high nutritional value and market demand. Aquaculture of magur is constrained by non-availability of seed due to problems in induced breeding. The problem in breeding is associated with males that they do not ooze out the milt even after the hormonal injection so the male need to be sacrificed each time. The better understanding of endocrine control at molecular level can help to find out its solutions. The current work is focused on steroidogenic acute regulatory protein (StAR) gene which plays an important role in steroidogenesis. The StAR gene was amplified from the testis of *C. batrachus* and cloned in expression vector pcDNA4/HisMax A. The functionality of the StAR gene construct was confirmed by transfecting in SSN-1 cell line. The SDS-PAGE and western blot analysis with His tag antibody showed that a recombinant protein of 30kDa was form inside the cells. The construct was conjugated with chitosan nanoparticles which had diameter of 135.4 nm and zeta potential 17.7 mV. The ability of the chitosan nanoparticle to form a complex with the construct (pcDNA4-StARorf) and its stability against endonucleases degradation was confirmed by gel retardation assay. The construct could be amplified from different tissues like testis, brain, head kidney and muscles after the intramuscular delivery of the construct on 5th day. The effect on selected reproductive genes viz. CYP11A1, CYP17A1, 3-β-HSD, 17-β-HSD and p450 aromatase (cyp19a1) was analysed by real time PCR which showed significant increase in expression level of all selected genes in nano-conjugated administered group compared to control. There was no significant change in the expression in the fish treated with naked construct. The increase in 11-Ketotestosterone and 17β-estradiol hormones level and changes in histology of testis tissue like increase in primary spermatogonial cells and spermatozoa in the seminiferous tubules in nano-conjugated treatment group showed the efficacy of chitosan conjugated construct and its role in reproduction. The present study is the first report on delivery of chitosan conjugated StAR gene construct in *C. batrachus.*

CONTENT

1. **INTRODUCTION** **1-3**

2. **REVIEW OF LITERATURE** **4-16**

2.1. Constraints with Induced Breeding in *Clarias batrachus* 4

2.2. Reproductive behaviour 5

2.3. Endocrine control in male fishes 5

2.3.1. Testis 5-6

2.3.2. Androgenic steroid hormones 5

2.3.3. Function of androgens 6

2.4. Different genes and their roles in fish reproduction 6-7

2.4.1. Cytochrome P450scc (CYP11A1) gene 7-8

2.4.2. Cytochrome P450 17a-hydroxylase, 17, 20-lyase gene 8-9

2.4.3. 3β-hydroxysteroid dehydrogenase (3β-HSD) gene 9

2.4.4. 17β-hydroxysteroid dehydrogenases (17β-HSD) gene 9-10

2.4.5. Cytochrome P450 aromatase gene 10

2.5. Steroidogenic acute regulatory protein (StAR) 10-11

2.5.1. Mutation in StAR gene 11

2.5.2. Maturation and activity of StAR protein 11-12

2.5.3. StAR family 12

2.5.4. Transcription regulation of StAR 13

2.6. Gene delivery methods 14

2.7. Chitosan for development of nanoparticles and gene delivery 14-16

3. **MATERIAL AND METHODS** **17-33**

3.1. Site of Experiment 17

3.2. Chemicals, Reagents and Kits 17

3.3. Equipment and Laboratory wares 17-18

3.4. Bacterial strains and vector 18

3.4.1.	Bacterial strains	18
3.4.2.	Culture conditions	18
3.4.3	Vector/Plasmid	18
3.5.	Tissue collection and preservation	18
3.6.	Nucleic Acid Isolation	19
3.6.1.	Total RNA isolation	19
3.6.2.	Quantification of RNA	19
3.6.3.	Gel electrophoresis of RNA	19-20
3.6.4.	DNase Treatment	20
3.6.5.	Reverse Transcriptase PCR (RT - PCR)	20
3.7.	Primers	20-22
3.8.	PCR Amplification of Target Transcripts	22
3.9.	Agarose gel electrophoresis of the PCR product	23
3.10.	Elution of PCR product from Gel	23
3.11.	T/A Cloning	23-24
3.11.1.	Ligation	24
3.11.2.	Transformation	24
3.11.3.	Analysis of recombinant clones	25
3.11.3.1.	Blue white selection	25
3.11.3.2.	Colony PCR	225
3.12.	Recombinant Plasmid DNA isolation	25-26
3.13.	Restriction enzyme digestion of recombinant plasmid	26
3.14.	Sequencing	26
3.15.	Formation of StAR Expression Constructs	26-27
3.16.	Endofree Plasmid Isolation	27
3.17.	*In vitro* checking of expression construct (pcDNA4-StARorf)	27
3.17.1.	SSN-1 cell culture	27

3.17.2. Harvesting cells by trypsinization 27-28
3.17.3. Passaging 28
3.17.4. Viability testing 28
3.17.5. Transfection 28
3.18. PAGE and Western blotting 28-29
3.19. Preparation of Chitosan Nanoparticles 29
3.19.1. Characterization of chitosan Nanoparticles 29-30
3.19.2. Nano-conjugation of the pcDNA4-StARorf with chitosan 30
3.19.3. Confirmation of DNA conjugation with nanoparticles 30
3.20. *In vivo* Experiments 30
3.20.1. Experimental animals 30
3.20.2. Experimental Design 30-31
3.20.3. Collection of Tissues 31
3.21. Nucleic acid isolation 31
3.21.1. Genomic DNA Isolation by Phenol-Chloroform Method 31-32
3.21.2. Total RNA isolation 32
3.21.3. DNase Treatment 32
3.21.4. cDNA synthesis 32
3.22. Analysis of Gene Expression 32
3.22.1. Tissue distribution study 32
3.22.2. Gene expression by Real time PCR 32-33
3.22.3. Histology 33
3.23. Analysis of data 33
4. RESULTS 34-52
4.1. Isolation of total RNA 34
4.2. Designing of Primers 34
4.3. PCR amplification of StAR gene 34

4.4. Cloning and screening of the recombinants 34

4.5. Construction of StAR gene expression plasmid 34-35

4.6. *In vitro* expression of pCDNA4-StARorf 35

4.7. Physicochemical characterization of Chitosan nanoparticles 35

4.7.1. Conjugation of chitosan nanoparticles with pcDNA4-StARorf 35

4.8. Tissue distribution of pcDNA4-StARorf 35

4.9. Expression profile of different reproductive genes 35-36

4.10. Serum hormone level 36

4.11. Histological study of gonads 37

5. DISCUSSION 53-57

6. SUMMARY 58-60

7. REFERENCES 61-72

8. ABBREVIATIONS

9. APPENDIX I

LIST OF FIGURES

Fig. No.	TITLE	PAGE. NO.
1.	Steroidogenesis production pathway	7
2.	RNA run on 1% agarose gel	38
3.	PCR amplification of StAR ORF of amplicon size 858bp	38
4.	Colony PCR of StAR fragment from recombinant clones	39
5.	pcDNA4-StARorf construct designed for *in vivo* expression	39
6.	Restriction enzyme digestion of pcDNA4-StARorf construct	40
7.	BLASTn analysis of sequencing result	40
8.a	SDS-PAGE analyis of cells transfected with pcDNA4-StARorf	41
8.b	Western blotting analysis	41
9.a	Mean diameter and poly dispersity index of chitosan nanoparticles	41
9.b	Zeta potential of chitosan nanoparticles	42
10.	Gel retardation assay	42
11.	Tissue distribution of pcDNA4-StARorf in 5 different tissues of *C. batrachus*	43
12.	Melting curve of different housekeeping genes	43-44

13.1 Fold change in expression and melting curve of cyp11a1 gene 45

13.2 Fold change in expression and melting curve of cyp17a1 gene 46

13.3 Fold change in expression and melting curve of 3-β-HSD gene 47

13.4 Fold change in expression and melting curve of 17-β-HSD gene 48

13.5 Fold change in expression and melting curve of P450 aromatase gene 49

14. Time-points versus OD value bar graph of 11-ketotestosterone 50

15. Time-points versus OD value line graph of 11-ketotestosterone 50

16. Time-points versus OD value bar graph of estradiol 51

17. Time-points versus OD value line graph of estradiol 51

LIST OF TABLES

Table No.	PARTICULARS	PAGE. NO.
1.	List of primers used for PCR amplification	21

LIST OF PLATES

PLATE No.	PARTICULARS	PAGE. NO.
1.	Histological sectioning of testis tissue of *Clarias batrachus*	52
2.	Histological sectioning of liver tissue of *Clarias batrachus*	52

1. INTRODUCTION

The walking catfish, *Clarias batrachus* (Linnaeus, 1758) is popularly known as magur and distributed in Eastern and North Eastern India. It is also distributed in other countries such as Thailand, Philippines, Cambodia, Myanmar and China (Mahapatra, B. K. 2004). *C. batrachus* is an air breathing catfish that normally lives in slow-moving and often stagnant water in ponds, swamps, streams and rivers, flooded rice paddies or temporary pools which may dry up. It can also survive in harsh conditions with minimal dissolved oxygen content. This fish is most popular among the consumers because of its good taste, hardy nature, rugged shape and medicinal value (Hossain *et al.,* 2006; Debnath, 2011).

The fish becomes sexually mature at approximately one year of age (Talwar and Jhingran, 1991). The species exhibit mass migration in rainy season and spawning events. Adhesive egg masses containing as many as 1,000 eggs are laid in nesting hollows prepared by the breeding pair. Egg masses are found on aquatic vegetation or within other suitable nest sites. They are guarded by the males until they hatch (Courtenay *et al.,* 1974; Courtenay and Hensley, 1980). The female, leaving care of the eggs to the male, guards the area around the nest. Embryonic development within the egg is rapid. Embryos hatch out in approximately 30 hours. For the first two days after hatching, parents still remain by the nest to protect the fry. At this stage, the fry has egg-yolk sac and for the first two to three days after hatching they use energy from it (Rao *et al*., 1995).

Aquaculture of magur is constrained by non-availability of seed with natural collections diminishing considerably and its complex reproductive behavior making induced breeding a complicated process requiring sacrificing of the male. Development of cost-effective and protractible induced breeding practices is imperative to promote aquaculture of fish (Muir, 2005). The *C. batrachus* female can be stripped and eggs can be obtained after inducing either with synthetic hormone or hypophysation. The males, however, do not readily ooze milt and so dissection of testes becomes necessary for obtaining milt. This reproductive dysfunction is not well understood and an effective induced breeding of *C. batrachus* is possible only when its endocrine regulatory pathway is well studied (Mazumdar *et al.,* 2007).

Steroid hormones play a crucial role in the regulation of growth, development, differentiation, reproduction and several other functions in vertebrates. The biosynthesis of steroids depends on the delivery of the common precursor, cholesterol, from the cytoplasm to the inner mitochondrial membrane in which it is converted to pregnenolone by the action of the cytochrome P450 side-chain cleavage enzyme (P450scc). However, cholesterol cannot cross the aqueous layer between the outer and inner mitochondria membranes from cytoplasm and is delivered by a sterol transfer protein, steroidogenic acute regulatory protein (StAR) (Stocco *et al.,* 2000). Now, it has been accepted that the true rate-limiting step in steroidogenesis is the delivery of cholesterol across mitochondrial membrane. StAR include the C-terminal START domain, which contains hydrophobic residues that contribute to forming a hydrophobic tunnel structure and phosphorylation sites. C-terminal-domain amino acids are known to be critical to the function of StAR in cholesterol transfer: Truncation leads to loss of steroid production (Arakane *et al.,* 1996). The steroidogenic acute regulatory protein is the member of the StAR-related lipid transfer domain (START) family. Regulation of steroidogenesis occurs in a tissue-specific manner and involves multiple signalling pathways, including protein kinase A (PKA) and protein kinase C (PKC), among others, and this appears to be conserved across most vertebrate species (Manna and Chandrala, 2006).

Attempts had been made to control the breeding process in catfish by supplying heterologous recombinant GnRH with some success as the reproductive genes are fairly conserved, but use of species specific gene sequences is very likely to be more effective by virtue of efficient binding to receptors (Evans, D.H., 1998). The specific sequences can also be valuable in expression studies using Real Time PCR. Novel delivery system for administration of DNA into the living system is a need for efficient and effective delivery. Among feasible delivery systems, chitosan nanoparticles hold pledge because of their ability to protect encapsulated nucleic acid-based systems, and to promote delivery of adsorbed DNA to respective receptors.

In this context, this work aims to accomplish the following objectives;

1. To develop chitosan conjugated StAR gene constructs in *Clarias batrachus*.

2. To determine the effects of chitosan conjugated StAR gene constructs on hormonal profile of *Clarias batrachus.*
3. To study the expression level of different reproductive genes after nano delivery of chitosan conjugated StAR gene construct by qRT-PCR.

2. REVIEW OF LITERATURE

Catfish (Order siluriformes) is a diverse and widespread fresh water group of Ostariophysan fish (Wright, 2009). Catfish is the most diverse fish order and the second or third diverse order among vertebrates; it represents 1 to 10 of the fish and 1 to 20 of the vertebrates (Dyk and Pieterse, 2008*).* Many species of catfishes have been introduced into aquaculture (Teugels, 1996). Understanding the biological processes especially the reproductive biology is the most important factor to improve the catfish culture. *Clarias batrachus* is native to south-eastern Asia and has been introduced into many places for fish farming (Lever, 1996). Walking catfish, 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 estuarine habitats, lakes, wetlands and is also known to inhabit medium to large rivers, swamps, ponds, ditches, flooded fields and rice paddies. It is also reported to occur in inter-coastal waterways of salinities up to 18 ppt. 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, walking catfish burrow into the sides of ponds and streams where they remain dormant until the spring rains begin (Masterson, J. 2007).

2.1. Constraints with Induced Breeding in *Clarias batrachus*

Unlike other species where a spermiating male spontaneously releases copious quantities of milt after injecting with gonadal steroid, the multi-lobed catfish testis does not produce a 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 acts as a major bottleneck for breeding and ultimately hamper production of seed for aquaculture.

2.2. Reproductive bbehavior

C. batrachus becomes sexually mature at approximately one year of age (Talwar and Jhingran, 1991). Spawning is triggered with the onset of monsoon which lasts from June to August in Eastern and North Eastern part of India. In the wild form, male fish show spotting in the dorsal fin but sexing can be done by examining the genital papillae of the fish. This is elongated and pointed in male and shorter and blunt in female. Female fish have much more rounded belly than males. Prior to spawning, body contact between the pair increases and they can be observed swimming side-by-side. In nature, the fish spawn in caves dug into the banks of rivers. The event itself can last as long as 20 hours, with the pair coming together many times above the spawning site. Six to seven thousands of eggs (Das, S. K. 2002) may be produced and these sink immediately, adhering to the substrate where they fall.

Following spawning, the female retreats and the male assumes protection of the brood, swimming tirelessly above the nest to guard the predators (Courtenay *et al.,* 1974; Courtenay and Hensley, 1980). It may also be that the currents produced by his unceasing movements assist in the development of the eggs. The eggs hatch in 24-36 hours, at which point the female again begins to assist in brood care, assuming responsibility for guarding the perimeters of the territory. The male remains at the nest. After around another 48 hours, the adults begin to lose interest in the fry and 72 hours later the young become free swimming.

2.3 Endocrine control in male fishes

2.3.1 Testis

The testes of teleost fishes show greater morphological variation than in other vertebrates (Callard *et al.*, 1978). In most cases, testes are a pair of elongated structures composed of branching seminiferous tubules embedded in the stroma. The testis consists of thin-walled tubules or lobules that contain spermatogonia which are endodermal in origin. Leydig cells or interstitial cells are the large polygonal cells located within the inter-lobular spaces, produce androgen (Guraya, 1976). Hurk et al. (1978) made an ultra structural and enzyme cytochemical study of the testis in rainbow trout and reported that the Leydig cells are the main source of steroids and the steroidogenic activity. It is at peak when the testes are mature and new spermatogonia are being formed. And also observe that sertoli cells have enzymes involved in steroidogenesis when males are in spermiation. Guraya, 1976 had observe that when spermatozoa are stored in the lumen of vas deferens the stromal cells around the vas deferens epithelium become steroidogenic.

2.3.2 Androgenic steroid hormones

11-ketotestosterone, was first isolated and identified in the plasma of sockeye salmon, *Oncorhynchus nerka* (Idler *et al.,* 1960). The 11-ketotestosterone is associated with the process of spermiation, development of secondary sexual characteristics, and regulation of male reproductive behavior in most teleost species (Schultz *et al.*, 2005). The androgenic activity of 11-ketotestosterone is 10 to 17 times that of testosterone and it is considered to be the true androgenic hormone in fishes (Tamaoki, 1980).

2.3.3 Function of androgens

The three primary steroid hormones that regulate mating behavior in teleost fish are 11-ketotestosterone, Testosterone and Estradiol. While these steroids are not always necessary for the expression of reproductive behaviors, they modulate neural pathways controlling reproductive behavior, resulting in increases or decreases in motivation (Gonçalves and Oliveira. 2010), suggesting that these sex steroids may play a role in species recognition mechanisms. 11-ketotestosterone is the primary androgen regulating male mating behavior in teleost fish reviewed in (Borg B. 1994). Androgens affect spermiation (the final stage of spermatogenesis) by binding to receptors on Sertoli cells (Miura *et al*., 1996). 11-KT specifically stimulates spermatogenesis in numerous fish platyfish, *Xiphophorous maculatus* (Schreibman *et al.*, 1986); catfish, *Clarias gariepinus* (Cavaco *et al.*, 1998).

2.4 Different genes and their roles in fish reproduction

In teleosts, the levels of steroids are critical for sexual development and hence, expression of steroidogenic enzyme genes and specific substrate availability are indispensable for gonadal steroidogenesis (Rajakumar *et al*., 2014). Biosynthesis of steroid hormones from cholesterol involves a cascade of steroidogenic enzymes. There are many genes which are involved in steroidogenesis pathway but in this study specific genes are selected that includes Cyp11a1, Cyp17a1, 3β-hydroxysteroid dehydrogenase (3β-HSD), 17β-hydroxysteroid dehydrogenase (17β-HSD) and Cytochrome P450 aromatase (cyp19a1b). The role of these genes are described below in following Fig. 1.

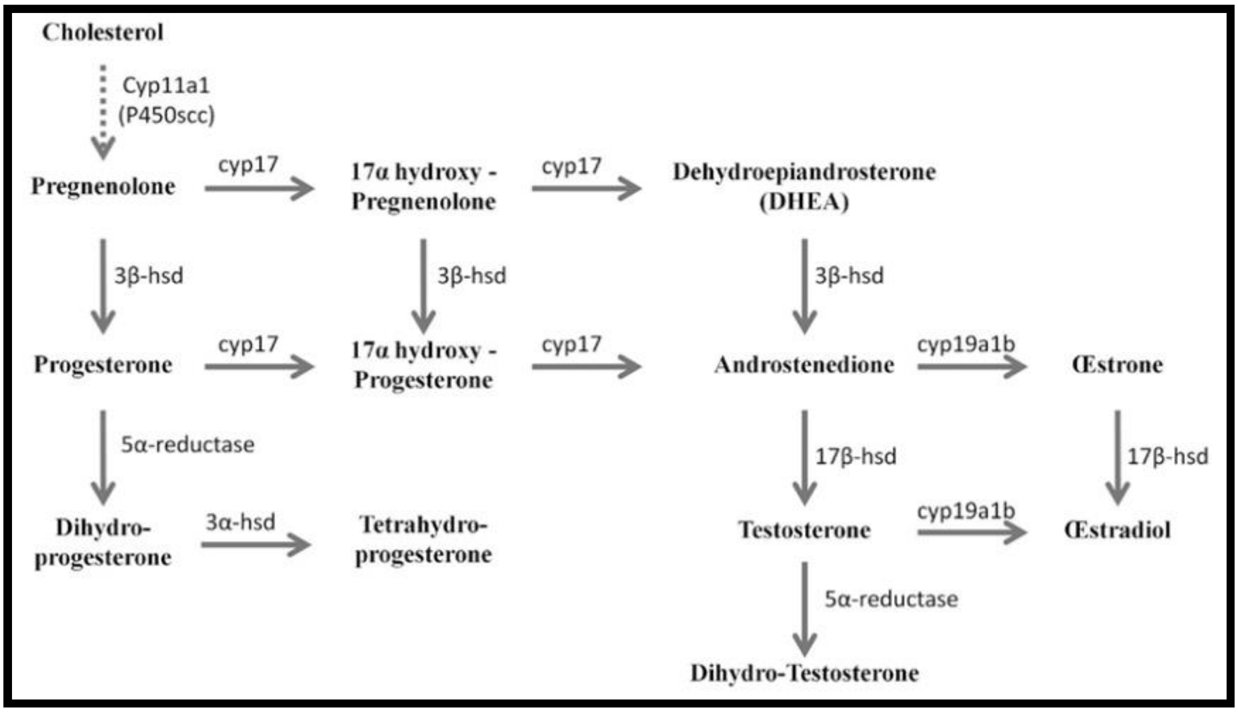


Fig. 1 The role of different genes involved in steroid hormone production (Source-Diotel *et al*., 2011)

2.4.1 Cytochrome P450scc (CYP11A1) gene

The P450scc cholesterol side-chain cleavage enzyme involved in the biosynthesis of pregnenolone, a sole precursor for all important steroids like estradiol-17β (E2), testosterone (T) and 17α,20β-dihydroxy-4-pregnen-3-one (DHP) and corticosteroids. This enzyme is located on the matrix side of the inner mitochondrial membrane and is a part of electron transport system (Chung *et al*., 1997). Movement of cholesterol and further conversion to pregnenolone are the presumed rate-limiting step of the steroidogenic pathway (Stocco, 2000) and thus Cyp11a1 occupies a key regulatory part in the biosynthesis of steroids. One of the important factors which regulate the activity of Cyp11a1 is the availability of its substrate, cholesterol. Cholesterol is stored in the lipid droplet away from the mitochondria and hence it has to be transported to the inner mitochondrial membrane for steroidogenesis. This step is very slow and requires the facilitation of the steroidogenic acute regulatory (StAR) protein (Stocco and Clark, 1996).

In zebrafish, duplicate copy of cyp11a gene, cyp11a2, was found which shares 85% identity with cyp11a1 in catfish, (Goldstone *et al.,* 2010; Parajes *et al*., 2013), However in C. batrachus, only one transcript was found. The cloned catfish cyp11a1 showed considerable homology with cyp11a1 of channel catfish (92%) and rainbow trout (76%). Expression of cyp11a1 was higher in the testis, brain, head kidney, muscle and ovary signifying its main role in steroidogenic tissues and additional role in non-steroidal tissues as well (Rajakumar *et al*., 2014). Developing and mature testes showed higher cyp11a1 expression than the ovary of corresponding age group. Interestingly, cyp11a1 expression is sexually dimorphic during mouse embryogenesis and is apparent at embryonic day 12.5 in the testis, while, it is not expressed in the developing ovary (Hatano *et al.,* 1994; Ikeda *et al*., 1994). The ubiquitous and sex specific expression was found in *O. bonariensis* (Blasco *et al*., 2010).

2.4.2 Cytochrome P450 17a-hydroxylase, 17, 20-lyase gene

Cytochrome P450 17a-hydroxylase, 17, 20-lyase (Cyp17a1) is a key steroidogenic enzyme essential for cortisol production in adrenal tissues and for the production of sex steroids in gonadal tissues. The Cyp17 enzyme possesses both the 17a-hydroxylase and 17, 20-lyase activities. The 17a-hydroxylase activity of Cyp17 converts pregnenolone to 17a-hydroxypregnenolone and progesterone to 17a-hydroxyprogesterone. The 17, 20-lyase activity is required for the production of sex steroids since it cleaves the C17, 20 bond to convert 17a-hydroxypregnenolone to dehydroepiandrosterone or 17a-hydroxyprogesterone to androstenedione, which is an important precursor for the production of estrogens and 11-oxygenated androgens (Hinfray *et al*., 2011).

In Asian swamp eel gonads, the cyp17 gene generates four isoforms of Cyp17 protein by alternative splicing and polyadenylation processes (Cheng *et al*., 2003). In tilapia and medaka fish, two Cyp17 protein isoforms (Cyp17-I and Cyp17-II) encoded by two different genes (cyp17a1 and cyp17a2) were identified (Zhou *et al*., 2007; *Zhou et al*., 2007b). The Cyp17-I isoform showed both the hydroxylase and lysate activities while the Cyp17- II isoform showed only the lysate activity (Zhou *et* *al*., 2007b). An in-silico search revealed the existence of two different cyp17 genes in the genomes of fugu, stickleback, tetraodon, and zebrafish (Hinfray *et al*., 2011). In catfish and zebrafish, cyp17a1 was expressed predominantly in gonadal and adrenal tissues but expression was also found in brain, liver, kidney, gills, heart, muscle, and intestine (Sreenivasulu *et al*., 2009a; Yajun *et a*l., 2004).

2.4.3 3β-hydroxysteroid dehydrogenase (3β-HSD) gene

Among all the enzymes required for biosynthesis of steroid hormones, 3β-hydroxysteroid dehydrogenase/Δ5–Δ4 isomerase catalyzes the dehydrogenation and isomerization of Δ5 steroids to Δ4 steroids. The enzyme is essential for the biosynthesis of most steroid hormones. 3β-HSD catalyzes pregnenolone, 17 hydroxypregnenolone, dehydroepiandrosterone and androstendiol to progesterone, 17α-hydroxyprogesterone, androstenedione and testosterone, respectively. Recently, recombinant 3β-HSD proteins from several mammalian species have been characterized (Bain *et al.*, 1991; Lorence *et al.*, 1990 and Lorence *et al*., 1991). Multiple forms of 3β -HSD proteins are present in mammals but in rainbow trout only single transcript is present (Kobayashi *et al.*, 1996).

2.4.4 17β-hydroxysteroid dehydrogenases (17β-HSD) gene

Steroid hormones act via specific receptors, which activate gene transcription. Hydroxysteroid dehydrogenases (hsd) converting steroids at positions 3, 5, 11, 17 and 20 of the steroid backbone are responsible for the pre-receptor regulation of steroid hormone action (Nobel *et al*., 2001; Morris *et al*., 2003). The 17β-hydroxysteroid dehydrogenases (17β-HSD) are key enzymes involved in the formation (reduction) and inactivation (oxidation) of sex steroids. Several types have been found in vertebrates including fish, as well as in invertebrates like *Caenorhabditis elegans, Ciona intestinalis and Haliotis diversicolor* supertexta (Ramos *et al*., 2014). Fourteen types of mammalian 17β-HSD have been described or annotated in public databases, but only 12 have orthologues in humans (Lukacik *et al*., 2006). 17 β -HSD type 1 (HSD17B1) catalyzes the reduction of estrone to estradiol, while type 3 (HSD17B3) performs the conversion of androstenedione to testosterone (Mindnich *et al.*, 2004).

The activation of the steroid signalling pathway is controlled by the bioavailability of sex hormones. The last steps of steroid synthesis and their primary metabolism are catalyzed by 17beta-hydroxysteroid dehydrogenases (17β-HSD). These enzymes convert inactive 17-keto-steroids into their active 17β-hydroxy-forms or vice versa. They differ in tissue distribution, catalytic preferences, substrate specificity, subcellular localization and mechanisms of regulation (Sivik *et al*., 2012).

2.4.5. Cytochrome P450 aromatase gene

Cytochrome P450 aromatase (P450arom) is the steroidogenic enzyme responsible for the conversion of androgen to estrogen. In mammals, there is only a single copy of the aromatase gene, CYP19A1, except in pigs, which have multiple copies of the CYP19A1 gene and in teleosts, two separate genes, named cyp19a1a and cyp19a1b, have been identified (Jeng *et al*., 2012). The gonads and the adrenal glands are the main established sites for P450 aromatase expression. In teleost fish, P450 gene expression has been studied predominantly in the gonads and the cDNA has been isolated from the ovary or testis in the Japanese medaka *Oryzias latipes*, rainbow trout *Oncorhynchus mykiss* (Sakai *et al.,* 1992), channel catfish *Ictalurus punctatus*, Japanese eel *Anguilla japonica* (Kazeto *et al.,* 2000) and *Clarias batrachus* (Dhalongsaih *et al., 2013).* In vertebrates, this enzyme is expressed in various tissues and plays an important physiological function by regulating estrogen biosynthesis (Matsumine *et al.,* 1986). Also, modulation of the activity of aromatase is strongly associated with sex reversal that occurs as a natural part of the life cycle for a number of different fish species (Guiguen *et al., 2001*). Recently, several reports have described the expression of P450arom during sexual differentiation in many species. Genetically female flounder larvae at high water temperature caused suppression of P450arom gene expression in the gonad and resulted in sex reversed males (Kitano *et al.*, 2000).

2.5 Steroidogenic acute regulatory protein (StAR)

Production of different classes of steroids occurs from a common precursor, cholesterol and involves a battery of oxidative enzymes (Payne and Hales, 2004). The first committed step in steroid hormone biosynthesis is the conversion of cholesterol to pregnenolone, which occurs in mitochondria by the action of P450 side chain cleavage enzyme (P450scc). However, cholesterol cannot cross the mitochondria from cytoplasm and is delivered by a sterol transfer protein, steroidogenic acute regulatory protein (StAR) (Stocco, 2000). Now, it has been accepted that the true rate-limiting step in steroidogenesis is the delivery of cholesterol across mitochondrial membrane. Therefore, StAR is indispensable for mediating cholesterol transfer vis-à-vis steroidogenesis.

2.5.1 Mutation in StAR gene

Lin *et al.* (1995) identified the mutations in StAR gene during congenital adrenal hyperplasia (CAH), a condition in which cholesterol and cholesterol esters accumulate in the steroidogenic tissues and the newborn fails to synthesize adequate levels of steroid hormones. It is an autosomal recessive disorder characterized by impaired adrenal and gonadal steroidogenesis and male pseudo hermaphroditism coupled with characteristic lipid deposits within the steroidogenic tissues (Bose *et al*., 1996). Three unrelated patients with lipoid CAH were shown to carry mutations in the StAR gene causing production of truncated, non-functional StAR proteins (Lin *et al*., 1995). Caron *et al*., (1997) shows that StAR knockout mice exhibit the phenotypic mirrors of human lipoid CAH in which the absence of StAR is lethal for mice, with some animals dying shortly after birth and others dying approximately 1 week after birth. These StAR knockout mice exhibit histopathological changes. The ovaries, which do not produce steroid hormones in significant amounts before puberty, do not exhibit detectable abnormalities, whereas the adrenal cortex and testes are clearly abnormal.

2.5.2 Maturation and activity of StAR protein

The StAR protein has an N-terminal sequence that directs the immature protein into the mitochondria where it is processed to yield the mature protein (Stocco *et al*., 1996). The N terminus of the StAR immature protein is not essential for the steroidogenic activity of StAR. Arakane *et al.* (1996) find out that a StAR mutant lacking the first 62 N-terminal amino acid residues is as steroidogenically active as full-length protein when expressed in COS-1 cells in conjunction with the human cholesterol side chain cleavage system. Further, Arakane *et al.* (1998) find out that recombinant N-62 StAR protein is incapable of being imported into mitochondria but stimulates pregnenolone synthesis by mitochondria isolated from bovine corpus luteum. These observations clearly interpreted that StAR acts on the outer mitochondrial membrane to promote cholesterol delivery to cytochrome P450ssc. The mechanism by which StAR enhance the substrate delivery to the cholesterol side chain cleavage enzyme has investigated by the so many researchers and no mitochondrial receptor has yet been identified for the StAR protein. StAR promotes cholesterol but not phosphatidylcholine transfer, distinguishing its activity from other known lipid transfer proteins (Kallen *et al*., 1998).

2.5.3 StAR family

The steroidogenic acute regulatory protein (StAR) is a member of the StAR-related lipid transfer domain (START) family. It is critical to regulate steroidogenesis in vertebrates. The steroidogenic acute regulatory protein (StAR)-related lipid transfer (START) domain is a protein domain spanning ~210 residues (Ponting and Aravind, 1999). Members of the START domain family have been shown to bind different ligands, such as sterols (e.g., StAR or STARD1) and lipids (e.g., PCTP or STARD2), and exhibit enzymatic activity. START domain is evolutionarily conserved in plants and animals. Fifteen START domain containing proteins (STARD1–STARD15) have been identified in humans (Soccio and Breslow, 2003). The crystal structures of three of these fifteen have been solved, revealing a conserved ‘helix-grip’ fold that forms an inner tunnel wide enough to accommodate the hydrophobic lipid (Tsujishita and Hurley, 2000; Roderick *et al*., 2002). The identity of the lipids that bind each START domain is known for only a few members of the family. However, only a very few START homologs have been reported in teleost fishes, which are the largest and most diverse group of vertebrates. The availability of sequenced and assembled genomes of zebrafish (*Danio rerio*), fugu (*Takifugu rubripes*) (Aparicio *et al*., 2002), Green Spotted Puffer (*Tetraodon nigroviridis*) (Jaillon *et al*., 2004), medaka (Kasahara *et al*., 2007) and three-spined stickleback has provided an opportunity for the genome-wide screening of START homologs and comparative analysis in teleost fish species. Through genome-wide screening, Teng *et al*. (2013) identified and annotated 91 START genes in 5 teleost species. Eighteen in Green Spotted Puffer, 3-spined stickleback, fugu, 21 in zebrafish, and 16 in medaka. The length of STARTs in teleosts ranged from 198 to 1928 amino acid residues, and the number of exons ranged from 5 to 19. In teleosts, about half of the START domain containing proteins (48/91) are multi-domain proteins.

2.5.4 Transcription regulation of StAR

Regulation of steroidogenesis occurs in a tissue-specific manner and involves multiple signalling pathways, including protein kinase A (PKA) and protein kinase C (PKC) and this appears to be conserved across most vertebrate species (Aesoy *et al*., 2002; Manna *et al*., 2005). ACTH is an upstream regulator of cAMP production, induces StAR mRNA expression in rainbow trout and eel (Hagen *et al*., 2006). Nunez *et al.* (2007) have find out that exogenous exposure of Atlantic croaker ovarian follicles to hCG robustly stimulates gonadal StAR mRNA expression.

StAR gene expression is cAMP-dependent and its regulation involves the transcriptional induction but the StAR gene promoter lacks a consensus cAMP response element (CRE) (Waterma, 1994) which suggest an alternating mechanism of StAR gene regulation. The mechanism of cAMP signalling at the gene level is mediated through an eight-base pair (bp) palindromic sequence (5'-TGACGTCA-3') which is referred to as the CRE (Meyer and Habener 1993). Sassone-Corsi (1995) find out that the 5'-TGACG part of the palindrome is highly conserved by characterising several CRE-binding factors, including the cAMP response-element binding protein (CREB), the CRE modulator protein (CREM) and activating transcription factor (ATF-1). Transcriptional activation of the StAR gene has also been demonstrated by multiple transcription factors including SF-1; however, the mechanism of this regulation is still not clearly understood. Manna *et. al*. (2003) have find out three CRE half-sites located in the proximal region of the mouse StAR gene and seen that the CREB/CREM and SF-1 all interact and cooperate to regulate StAR gene transcription.

The human StAR gene promoter has at least two cis elements that govern basal and cAMP-regulated gene expression. One of these elements (the distal element) is a consensus binding sequence for the orphan nuclear receptor transcription factor, steroidogenic factor l (SF-1), the other (the proximal element) is a related motif. Deletion or mutation of the distal and proximal cis elements individually substantially reduces SF-l-supported StAR promoter activity. The distal site binds SF-1 with high affinity, whereas the proximal site binds SF-1 with lower affinities. These findings demonstrate a requirement for SF-1 for human StAR gene expression (Sugawara *et al*., 1997).

2.6 Gene delivery methods

Gene therapy is a promising approach to treat intractable and refractory diseases at the genetic level. Basically, in gene therapy, target gene expression is induced by delivering foreign genes. Down regulation of target gene expression or gene silencing can also be performed using miRNA, siRNA or shRNA expression vectors (Boudreau *et al.*, 2012). There are different methods of gene delivery like Intradermal, intramuscular, intra-peritoneal, oral gene delivey, immersion in water etc. Corbeil *et al.* (2000) injected pIHNVw-G vaccine in 1.8 g rainbow trout fry and the results of the virus challenge showed that the intramuscular injection and the gene gun immunisation induced protective immunity in fry, while intraperitoneal injection provided partial protection.

Rainbow trout with an average size of 5 g were injected with 2 µg of plasmid intramuscularly (i.m.) and intraperitoneally (i.p.) The injection volumes were 20 µl (i.m. injection) or 40 µl (i.p. injection). At day 5, the fish were killed and luciferase expression in the muscle cells was 6.8 fold higher than control (Romøren *et al*., 2004). Rainbow trout with an average size of 60 g had showed 4.1 fold increase in luciferase level expression when injected with 5 µg of plasmid intramuscularly (i.m.) at 5th day. The direct injection of pCAT-Control into the muscles of young growing carp of around 10 cm in length, CAT activity obtained was more than 90%, compared to 0% in non-injected muscles (Ekkehard *et al*., 1991). The intramuscular injection of 50 µg of DNA in rainbow trout of 15cm induced significantly higher levels of luciferase expression (t-test, $p < 0.05$). A general trend of increasing mean luciferase activity was observed in fish sacrificed at 10, 30 and 60 d after the injection of pCMVtkluc plasmid (Gomez *et al*., 1996).

2.7 Chitosan for development of nanoparticles and gene delivery

Gene delivery can particularly be used for the treatment of diseases by the insertion of genetic materials (DNA and RNA) into mammalian cells either to express new proteins or to prevent the expression of existing proteins (Saranya *et al*., 2011). Viral gene delivery systems have been found to have high transfection efficiency for a wide range of cell types but they have some disadvantages such as virtually induced inflammatory responses and oncogenic effects (Simon *et al*., 1993). To overcome these problems, non-viral gene delivery systems using biopolymers have been developed to deliver genes with minimal host immune responses (Romoren *et al.,* 2002; Bozkir and Saka, 2004; Kumar *et al*., 2008). Cationic polymers were found to be promising delivery systems among the non-viral delivery systems (Garnett, 1999). Among the polymers used to encapsulate pDNA are poly (D, L-lactic-co-glycolic) acid (PLGA) (Capan *et al.,* 1999), gelatine (Leong *et al.*, 1998) and chitosan (Hirosue *et al.*, 2001; Bozkir and Saka, 2004).

Chitosan is a natural biodegradable, biocompatible and non-toxic biopolymer extracted from the shells of crustaceans. Mumper *et al*. (1995) have used the chitosan for the first time to deliver the plasmid in the cellular system. Chitosan and its derivatives have been examined extensively for medical and pharmaceutical applications especially in artificial organs, targeted drug delivery, drug transport, protein delivery and gene transfer (Pan *et al*., 2002; Lavertu *et al.*, 2006; Wei and Zhang, 2007). Chitosan has favourable biocompatibility characteristics as well as the ability to increase membrane permeability, both in vitro and in vivo and can be degraded by lysozyme in serum (Aspden *et al.*, 1996). It has been extensively investigated for formulating carrier and delivery systems for therapeutic macro solutes, particularly genes and protein molecules primarily because positively charged chitosan can be easily complexes with negatively charged DNAs and proteins (Richardson *et al.,* 1999; Janes *et al.,* 2001). Chitosan can effectively bind DNA and protect it from nuclease degradation (Cui *et al*., 2001; Illum *et al.,* 2001). The application of DNA–chitosan nanoparticles has been studied widely for DNA transfection research which shows its usefulness for gene delivery (Romoren *et al*., 2002; Corsi *et al.*, 2003). Ramya *et al.* (2013) find that a DNA construct containing extra small virus antisense (XSVAS) gene of nodavirus encapsulated with chitosan nanoparticles (NPs) gave better protection to the animals against the pathogen, and survival percentage was significantly higher. The therapeutic efficacy of the nanoparticles is due to their ability to protect the therapeutic agent from degradation by lysosomal enzymes. Due to chitosan nanoparticles sub-cellular and sub-micron size, they can penetrate deep into tissues through fine capillaries and cross the fenestration present in the epithelial lining (Vinagradov *et al.*, 2002). This allows efficient delivery of therapeutic agents to target sites in the body. Further by modulating the nanoparticles characteristics, such as enzymatic degradation rate, size and surface charge density, we can control the release of therapeutic agent to achieve desired therapeutic level in target tissue for required duration (Panyam *et al*., 2003).

However, to date there is no study on chitosan nanoparticle conjugated StAR gene and its delivery in fishes.

3. MATERIAL AND METHODS

3.1. Site of Experiment

The molecular work was carried out in the Fish Genetics and Biotechnology laboratory, Central Institute of Fisheries Education, Mumbai. The wet lab experiment was carried out at Balabhadrapuram freshwater fish farm, CIFE Kakinada centre, Andhra Pradesh. The work on cell lines was carried out in the Cell Culture laboratory of the Aquatic Environment and Health Management Division of CIFE, Mumbai.

3.2. Chemicals, Reagents and Kits

Chemicals used in this study were generally of molecular biology grade. agarose, ethidium bromide, TAE buffer, LB agar and LB broth were purchased from Hi-Media, India. Ampicillin and ethanol were purchased from Amresco, USA. The 2x PCR master mix, nuclease free water, 6x gel loading dye, X-gal and IPTG were purchased from Fermentas, USA. Trizol reagent was purchased from Invitrogen. Taq DNA polymerase and restriction endonucleases such as *Bam*HI, *Eco*RI and *Hind*III were purchased from Fermentas, USA. T4 DNA Ligase was provided with the InsTAclone PCR Cloning kit (Fermentas, USA) and HOT FIREPol® EvaGreen® qPCR Mix Plus (ROX) (Solis Bio Dyne UK).

Kits used in this study were AffinityScript QPCR cDNA Synthesis Kit (Agilent Technologies, USA), Gel Extraction Kit (Agilent Technologies, USA), InsTAclone PCR cloning kit (Fermentas, USA), StrataPrep DNA Gel Extraction Kit (Agilent Technologies, USA), StrataPrep Plasmid Miniprep Kit (Agilent Technologies, USA), and EndoFree Plasmid Maxi Kit (Qiagen, USA).

3.3. Equipment and Laboratory wares

The equipment used in this study include PCR thermal cycler (Applied Biosystems, BioRad, USA, Takara, Japan); Nano particle size analyzer SZ-100 (Horiba scientific, Japan) Nanodrop 2000/2000c (Thermo Scientific, USA); refrigerated centrifuge (Heraeus, Germany); Deep Freezers (-20^{0}C) (HMG, India; Seimens, Germany); Deep Freezers(-80^{0}C) (Kaltis, USA); Inverted microscope, Horizontal gel electrophoresis apparatus (Bio-Rad, USA); shaking incubator

(ORBITEK, India), Gel documentation system (BioRad, USA); Millipore water purification system (Milli-Q, ElixTM, Millipore Corporation, Australia); tissue homogenizer (Tissue Tearor, USA) thermostatic water bath (ESCY, USA), electronic balance (OHAUS, USA), tabletop centrifuge 5430 (Eppendorf, Germany), incubator (Wadiegarfl India), Laminar Flow (Yorko Scientific Industries, India); microwave oven (Kenstar, India), autoclave (TOMY, Japan), micropipettes (Eppendorf, Germany) and UV transillummator (UVP, USA), Real time PCR (LightCycler® Roche, USA), 96-well low-profile semi-skirted PCR plate for the fast block of Life Technologies PCR and qPCR thermal cyclers (Thermoscientific, Germany) were used. The glass ware and plastic ware used in this study was from Borosil, India and Tarsons, India, respectively.

3.4. Bacterial strains and vector

3.4.1. Bacterial strains

The recombinase A deficient amber suppressing strain of *E. coli* DH5α, available at Molecular biology lab, Fish Genetics and Biotechnology division, Central Institute of Fisheries Education was used as host strain for cloning. The genotype of DH5α is supE44 ΔlacU169 (ψ80 lac Z ΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1.

3.4.2. Culture conditions

DH5α culture was maintained on LB agar plates and stored at 4°C for regular use. Sub-culturing was done every 20 days to maintain the cultures.

3.4.3. Vector/Plasmid

Vector used in this study include pTZ57R/T and pcDNA4/HisMax A. Plasmid pTZ57R/T having ampicillin resistance gene provided along with InsTAclone PCR Cloning kit (Fermentas, USA) was used for T/A cloning of the PCR amplified products. And pcDNA4/HisMax A was used for making expression construct.

3.5. Tissue collection and preservation

Clarias batrachus fishes are acquired from CIFA, Bhubaneswar. Testis and Brain were collected using sterile scissors and forceps, taking care to avoid contamination and stored in Qiagen’s RNAlater™ solution for RNA isolation.

3.6. Nucleic Acid Isolation

3.6.1. Total RNA isolation

Total RNA was isolated from brain and gonads of *C. batrachus* following Sambrook *et al*. (2001) with slight modifications. One hundred mg of sample was taken directly in 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 20-30s at room temperature. The homogenate was incubated for 5 min at room temperature to allow lysis and was centrifuged for 5 min at 10000 rpm. The aqueous phase was transferred to a fresh tube. To the lysate, 200 µL of chloroform (0.2 vol/ml Trizol™ reagent) was added and mixed by vigorous vortexing and incubated at room temperature for 10 min. The sample was centrifuged at 12,000 rpm for 15 min at 4°C. The supernatant was collected without disturbing the interface and transferred to a fresh 1.5 ml tube. The RNA was precipitated by adding 500µL of ultrapure isopropanol (0.5 vol/ ml Trizol™ reagent) for 10 min at room temperature and subsequent centrifugation at 12000 rpm for 15 min at 4°C. The precipitated RNA was washed twice with 1ml of 70% ethanol. The RNA pellet was vacuum dried for 10 minutes and dissolved in 30 µL of nuclease free water and stored at -80°C for further use.

3.6.2. Quantification of RNA

The concentration of the isolated RNA was measured using Nanodrop 2000/2000c (Thermo scientific, USA). Nucleic acid concentration was obtained directly in terms of ng/ µL along with the 260: 280 ratios. The ratio of the absorbance at 260 nm and 280 nm provided the estimate of the purity of the isolated RNA.

3.6.3. Gel electrophoresis of RNA

The integrity of the isolated RNA was checked by electrophoresing on 1% agarose gel. Appropriate amount of agarose (0.4 g) was added to 40 ml of 0.5 X TAE buffer and melted in a microwave oven till the solution became completely clear. The solution was cooled and 2 µL of 10% ethidium bromide was added and mixed thoroughly. The solution was poured into the comb fixed gel tray and allowed to solidify. The comb was removed carefully and the gel tray was placed in the gel electrophoresis buffer unit containing 0.5 X TAE buffer. Appropriate volume of RNA (1 µg) was mixed with 1 µL of 6X gel loading buffer and loaded into the gel slots. The unit was connected to power supply and the gel was run at 80 V for 1 hour. The gel was then taken out and documented in a Gel documentation unit (Bio-Rad, USA).

3.6.4. DNase Treatment

Total RNA isolated using Trizol™ reagent contains significant amounts of genomic DNA. Therefore the RNA was treated with 'RNase free' DNaseI (MBI Fermentas, USA) before cDNA synthesis. A mixture of 2.5 µg of total RNA, 1 µL of Buffer (10x) and 1 µL DNAse was taken in PCR tube made up to 10 µL by adding nuclease free water. The reaction mixture was incubated at 37°C for 30 min. The reaction was terminated by adding 1 µL of 25 mM EDTA followed by a 10 min exposure at 65°C.

3.6.5. Reverse Transcriptase PCR (RT - PCR)

The mRNA pool was converted to its complementary DNA using the AffinityScript QPCR cDNA Synthesis Kit (Agilent Technologies, USA) as per the manufacturer's instructions. Briefly, 1 µg of the DNase treated RNA sample, 10 µL of first strand master mix (2×), 3 µL of oligo- dT primers, 1.0 µl of AffinityScript RT/ RNase Block enzyme mixture was mixed with nuclease free water to make a final volume of 20 µL in a PCR tube and centifuged. This mixture was then incubated at 25°C for 5 minutes for primer annealing and incubated the reaction at 25°C for 5 minutes to allow primer annealing and then at 42°C for 15 minutes to allow cDNA synthesis. This reaction was terminated by incubating at 95°C for 5 minutes. The synthesized first strand cDNA was directly used in PCR to amplify the gene using specific primers.

3.7. Primers

Selected cDNA fragments were amplified using specific primers, which were designed using software Gene Runner V. 5.0 against the reported sequences from NCBI GeneBank. Several factors, such as primer length, % GC content, melting temperature, annealing temperature, secondary structures, etc. were taken into consideration while designing the primer sets. Appropriate linkers containing restriction enzyme recognition sequences were added to 5' and 3' ends of the primers other than the real time primers to facilitate directional cloning of the fragments into desired vectors.

Table1: List of primers used for PCR amplification

Primer name	Sequence 5’- 3'	Ta	Product length
StAR-BamH1-F	AAAGGATCCAGATGCTACCTGCAACTTTTAAGCTG	58	858
StAR-EcoR1-R	AAAAAGAATTCTCAGCAGGCCATTGCCTCCTC		
Cyp17a1-D-F1	GGGATTGTGGACACAGTTGCC	55	495
Cyp17a1-D-R1	GAGACTGGCCGGATTCGAAGCA		
P450aro-qRt-F	GCACCGCTCCGAGTTCTTC	60	120
P450aro-qRt-R	GAGGACCGGAGCCAAAGG		
Cyp11a1-qRt-F	AGCTGATGGCTGGAGGTGTAG	58	125
Cyp11a1-qRt-R	CAGTGCGAGCTGCAATGACT		
17βhsd-qRt-F	TCGTCCTCATCAGCCGTACTC	60	110
17βhsd-qRt-R	CCGATCCAAAGTCTGCAGAGA		
3βhsd-qRt-F	TGAGGGAGCCACAGACAAGAG	60	123
3βhsd-qRt-R	GCCAAAGCCAAGTGGTACCA		
qrtCYP17a1-F	GCTCCAGCACTTTCCAAATGAAG	60	119
qrtCYP17a1-R	ACGGAGTAGAGCATCCAACAGGTC		
CMV-F	GAATCTGCTTAGGGTTAGG	55	650
CMV-R	AATTTCGATAAGCCAGTAAGC		
β-actin F	GCACCCCGTCCTGCTTACTGAG	55	250
β-actin R	CCAGACGGAGGATGGCATGG		
M13-F	GTAAAACGACGGCCAGT	55	200
M13-R	CAGGAAACAGCTATGAC		

Primers were synthesized commercially by Bioserve and Eurofins Genomics Pvt. Ltd., Bangalore. The primers received in lyophilized form from the supplier were dissolved in 1X TE buffer (pH 7.0) as per the directions given by the supplier to get a stock solution with a concentration of 100 pmol/µl, which was diluted to a working concentration of 10 pmol/µl with nuclease free water.

3.8. PCR amplification of Target Transcripts

For amplification, StAR cds was amplified in sterile PCR tubes with a reaction volume of 20 µL using the following components.

Reaction components	Volume
10X buffer with $MgCl_2$	2.0 µL
10mM dNTP	0.5 µL
StAR-BamH1-F	0.8 µL
StAR- EcoR1-R	0.8 µL
cDNA	1 µL
Taq polymerase	0.25 µL
Water	14.65 µL
Total	20 µL

Amplification was carried out using the following PCR conditions in thermal cycler (Bio-Rad, USA).

PCR conditions	Temperature	Time	No. of cycles
Initial denaturation	94°C	4 minutes	1
Denaturation	93°C	45 seconds	35 cycles
Annealing	58°C	45 seconds	
Extension	72°C	1 minute	
Final Extension	72°C	7 minutes	1
Hold	4°C	Forever	

3.9. Agarose gel electrophoresis of the PCR product

Agarose gel electrophoresis of the amplified PCR product was carried out in 1% agarose gel. Weighed 0.4g of agarose powder and added to 40 ml of 0.5X TAE buffer and heated in a microwave oven till the solution became completely transparent. The solution was considerably cooled and 2 µL of 10% EtBr was added and then mixed thoroughly. The solution was poured into the gel tray fitted with combs and allowed to solidify. The comb was removed carefully and the gel tray was placed in the gel electrophoresis unit filled with 0.5X TAE buffer. The resultant PCR product was mixed with 2 µL, 6X gel loading buffer and loaded into the gel slots. In the first lane 1 µL of 100bp plus DNA ladder was loaded. The gel was run at 80-100V for 45 minutes. After that the gel was taken out and viewed in a transilluminator and documented in a Gel documentation unit (Bio-Rad, USA).

3.10. Elution of PCR product from the gel

The selected DNA band was excised from the gel and eluted using Gel Extraction kit (Agilent, USA). The excised gel was loaded in a micro centrifuge tube and weighed. Three volumes of DNA extraction buffer were added to 1 volume of the gel and the tubes were incubated at 50°C for 10 minutes in a thermostatic water bath. The tubes were vortexed every 2-3 minutes during the incubation. The samples were applied to the spin columns and centrifuged at 12,000 rpm for 30 seconds to bind the DNA to the column and the flow through was discarded. The column was washed with 0.75 ml of wash buffer to remove the buffer and other impurities from DNA. The column was centrifuged again to remove the residual wash buffer. Following this, the spin column was placed in a new sterile 1.5 ml micro centrifuge tube and 30 µL of elution buffer was added to the center of the column membrane. The spin column was allowed to stand for 5 minute and was centrifuged at 12,000 rpm for 1 minute. The resulting DNA fragment was checked on a 1 % agarose gel.

3.11. T/A Cloning

PCR products amplified by Taq polymerase have an extra single 3'- A overhang added to each strand of the PCR product. It allows these amplicons to be cloned into vectors that have been specifically designed to carry a T overhang on the complementary strand (called T/A vectors). The desired DNA fragments were cloned into pTZ57R/T vector using InsTAclone™ PCR Cloning Kit (Fermentas, USA). The resultant PCR product with ‘A’ overhang was mixed with the vector in 3:1 proportion of molar ends in 1X ligase buffer. Five units of T4 DNA ligase were added and the ligation mixture was incubated at 16°C overnight. Finally 3 µL of ligation mixture was transformed into *E. coli* DH5α competent cells.

3.11.1. Ligation

The insert was ligated into a pTZ57R/T vector provided with the InsTAclone PCR Cloning kit (Fermentas, USA). The ligation mixture was prepared in 20 µL total volume by adding the following components, 4 µL of 5X Ligation buffer, 3 µL of pTZ57R/T vector, 6 µL of purified PCR fragment, 1 µL of T4 DNA Ligase and made the final volume up to 20 µL with nuclease free water. The reaction mixture was mixed and incubated at 16°C overnight. The ligation mixture vector used for the transformation was DH5α strain of *E. coli.*

3.11.2. Transformation

Transformation was carried out by using InsTAclone PCR Cloning kit (Fermentas, USA) as per the protocol supplied by the manufacturer. DH5α strain of *E. coli* was inoculated into 5 ml of LB broth taken in a sterilized test tube and the culture was incubated overnight at 37°C in a shaking incubator. An amount of 1.5 ml of fresh C-medium was taken in a micro-centrifuge tube which was pre-warmed at 37°C. To this solution, 300 µL of the overnight culture was added and incubated at 37°C for 45 minutes in a shaking incubator at 150 rpm. The bacterial culture obtained was used for transformation. T-solution was prepared by mixing equal volumes of T-solution (A) with T-solution (B) and was kept on ice. The 1.5 ml of *E. coli* culture was centrifuged at 10,000 rpm for 1 minute. The supernatant was discarded and the cell pellet was re-suspended in 300 µL of T-solution and the tube was incubated on ice for 5 minutes. Again the cells were pelleted down at 10000 rpm for 1 minute and the supernatant was discarded. The cells were resuspended in 120 µL of T-solution and incubated on ice for 5 minutes. For 2 minutes 2.5 µL of ligation mixture was held in ice, to which 50 µL of the re-suspended cells were added and incubated on ice for 5 minutes. Finally the cells were spread plated on the pre warmed X-gal-IPTG and Ampicillin added LB agar plates and incubated overnight at 37°C in an incubator until the transformed bacterial colonies were visible.

3.11.3. Analysis of recombinant clones

3.11.3.1. Blue white selection

The primary confirmation of the clones was done by α-complementation and white colonies were selected from IPTG/X gal- Amp plates. The colonies that were fully or partially blue were discarded. The isolated white colonies were picked up to prepare a master plate. The confirmation of clone was done by colony PCR followed by RE digestion of the isolated plasmid.

3.11.3.2. Colony PCR

White colonies were screened for the presence of insert using colony PCR. Colony PCR was performed with gene specific primers for rapid confirmation of recombinant clones. The white colonies were picked individually using sterile disposable inoculation loop and suspended in 10 µL of TE. The PCR reaction mixture comprised of 10 µL of 2X PCR master mix, 1 µL each of 10 pmols M13 forward and reverse primers, 1 µL of colony suspended in TE solution and 7 µL nuclease free water. The PCR cycling conditions included an initial denaturation at 94°C for 4 min followed by 35 cycles of denaturation at 94°C for 30s, annealing at 55°C for 30s and extension at 72°C for 30 seconds. Final extension was done at 72°C for 5 min. The insert size was determined by running the PCR product on an agarose gel against a molecular size standard.

3.12. Recombinant Plasmid DNA isolation

Plasmid DNA from the recombinant clone was isolated using the StrataPrep Plasmid Miniprep Kit (Agilent Technologies, USA) as per the manufacturer’s protocol. A positive colony was picked from the master plate and grown overnight in 5 ml LB broth with 50 µL /ml ampicillin at 37°C in a shaking incubator. The bacterial culture was harvested by centrifugation at 12,000 rpm for 1 minute to obtain the cell pellet. The bacterial cells were then re-suspended in 100 µL of solution 1 by vortexing. Then 100 µL of solution 2 was added and mixed well by inverting the tube several times. After that solution 3 was added and mixed immediately by inverting the tube. This mixture was then centrifuged at 12,000 rpm for 5 minutes. The supernatant was pipetted out and transferred to the spin column with a collection tube. The spin column was centrifuged at 12,000 rpm for 1 minute and the flow through was discarded. Then, 750 µL of wash buffer was added to the spin column and centrifuged at 12,000 rpm for 1 minute. The flow through was discarded and the column was again centrifuged at 12,000 rpm for 1 minute to remove the residual wash buffer. The spin column was placed in a new collection tube and 30 µL of elution buffer was added to the center of the spin column and it was allowed to stand for 1 minute. Then the spin column was centrifuged at 12,000 rpm for 1 minutes and the recombinant plasmid DNA was eluted. The integrity of the isolated plasmid was checked on 1% agarose gel.

3.13. Restriction enzyme digestion of recombinant plasmid

Restriction digestion was performed to confirm the presence of the insert cloned in pTZ57R/T vector. An aliquot of the purified plasmid DNA was digested with BamH1 and *EcoRI* using conditions specified by the manufacturer. A total volume of 20 µL reaction mixture was prepared by adding 1 µg of purified plasmid DNA, 2 µL of 10x fast digestion buffer, 1 µL of BamH1 and *EcoRI* each and 15 µL of nuclease free water. This reaction mixture was incubated at 37°C for over night. The insert sizes were confirmed against a molecular size standard. All the inserts were confirmed by sequencing.

3.14. Sequencing

The confirmed recombinant plasmid was sequenced using ABI Big DYE terminator method (Eurofins, Bangalore, India). The sequence from the sequencer was obtained in a chromatogram. The data obtained in chromatogram was analyzed using Chromas LITE 201 software. The obtained sequence was having vector sequences attached to its flanking ends which were removed and BLASTn was done to confirm its identity.

3.15. Formation of StAR Expression Constructs

The full coding region (cds) of the *Clarias batrachus* StAR gene was amplified using the specific primers StAR-orf F/R (Table 1) designed to amplify the region between +57 to +914 bp of the gene. Linkers (BamH1 and EcoR1) were attached with the primers for directional and in-frame cloning. The amplicon was separated on agarose gel and the specific band was excised and purified from the gel. The fragment was digested using enzymes *BamH1* and *EcoR1* and cloned into similarly digested eukaryotic expression vector pcDNA4/ HisMax A under the control of CMV promoter. A positive clone was sequenced and confirmed to be in-frame with the ATG start codon. The construct was named pcDNA4-StARorf.

3.16 Endofree Plasmid Isolation

Endotoxins produced by the bacterial cells are generally co-purified with the plasmid DNA when conventional plasmid isolation procedures are followed. Therefore, for transfection in SSN-1 cell lines, plasmids were prepared using the Endofree plasmid purification kit (Qiagen, USA) following manufacturer's instructions. Plasmids used for direct injection into the animals were isolated using Endofree Plasmid Maxi Kit (Qiagen, USA) following manufacturer's instructions.

3.17 *In vitro* checking of expression construct (pcDNA4-StARorf)

For checking the expression of construct, the pcDNA4-StARorf construct was transfected into SSN-1 (Striped snakehead whole fry) cell line using the protocol described below.

3.17.1. SSN-1 cell culture

The SSN-1 cell line was available in Cell Culture laboratory of the Aquatic Environment and Health Management Division of CIFE. The cryovials containing cells preserved in liquid nitrogen were thawed and revived as per standard protocol. The cryovial from liquid nitrogen was shaken in a water bath at 37^oC and the cap slightly loosened to allow escape of any liquid nitrogen that might have entered. The thawed vial was wiped with 70% ethanol and then transferred to the laminar flow. The contents were gradually diluted 20-30 times by adding prewarmed L-15 Medium (Leibovitz) dropwise. The cells suspension were sedimented by centrifugation at 200g for 10 min. The supernatant was discarded and the cell pellet resuspended in L-15 Medium and inoculated into culture flasks and incubated O/N at 28^oC. The cells were observed for confluency and viability periodically.

3.17.2. Harvesting cells by trypsinization

The culture flask was observed under inverted microscope to check for contamination and cell density. Spent media was discarded and the cell monolayer was washed 2-3 times with trypsin. 0.5 ml of 0.5% Trypsin solution which had been pre-warmed to 37^oC was added and flask was gently shake till the most of cells were not deteched and the culture flask was checked under microscope to make sure that the cells were getting dislodged. After that 15 ml medium was added and mix with repeated pipetting. These cells were used for passaging and growing in 40 ml flask for subsequent transfection using Turbofect reagent.

3.17.3. Passaging

Cells from above were inoculated in T-25 flask, gently swirled for uniform mixing and incubated at 28^0c. The cells were passaged once a week when they became confluent.

3.17.4. Viability testing

Trypan blue stain which stains only dead or dying cells was used to determine the viability of the cells using a hemocytometer. Briefly, the harvested cells were re-suspended in PBS to a density of a minimum of 10^6cells/ml. To 0.1 ml of cell suspension, 0.5 ml of trypan blue (0.4% w/v) and 0.3 ml of PBS were added and mixed gently. After 2 min, 10 µl of the cell suspension was added to the hemocytometer chamber and cover-slipped. Stained and unstained cells were counted under a microscope. The unstained cells were inferred as viable cells and expressed as a percentage of the total number of cells.

3.17.5 Transfection

The transfection of the cell lines was done using TurboFect transfection reagent (Thermo Scientific, USA) following the manufacturer’s protocol. Four wells were transfected with 1 µg pcDNA4-StARorf in each well and two wells with 1µg pCDNA4/HisMaxA vector. One well each was transfected with 2 µg of pCDNAHisMax4A vector and the transfection reagent to rule out any contamination. The transfected plates were incubated at 28^0c and 48 h post transfection the cells were washed briefly with PBS and harvested in a 1.5 ml sterilized tube.

3.18. SDS-PAGE and Western blotting

For analysis of recombinant protein expression in cell line, SDS Poly-acrylamide gel was cast in a A A Hoefer apparatus assembled following manufacturer’s instructions. The resolving gel was prepared to 12% concentration and stacking gel to 5% concentration as outlined in table 4.4 and 4.5 of appendix I. The components were mixed in the order specified in the table.

The specific protein bands were identified from the total proteins separated on SDS-PAGE by western blotting. The bands separated on SDS-PAGE were transferred onto a PVDF membrane charged in methanol and cut to match gel size by the semi-dry blotting method. The membrane was blocked in 3% BSA overnight at 4°C and further incubated at room temperature for 1 h with shaking. The blocked membrane was washed thrice with PBS-Tween20 solution for a duration of 5 minutes each and treated with the primary antibody appropriately diluted with PBS for a duration of I hour. The membrane was further washed thrice with PBS-T followed by treatment with the secondary antibody for 1 h. The secondary antibody was diluted with PBS to a final concentration of 1:2,000. After treatment with the secondary antibody, the membranes were treated with HRP substrate solution (1% w/v Diaminobenzidine containing 0.001% H_2O_2) for 5 s to allow the development of colour at the specific band size. The reaction was terminated by the addition of distilled water. The membranes were dried and photographed using Bio-Rad Gel Doc™ XR^+ gel documentation system.

3.19. Preparation of Chitosan Nanoparticles

Chitosan nanoparticles were prepared based on ionic gelation method (Grenha *et al*. 2012) of chitosan and tri-polyphosphate (TPP) anion with little modification. Briefly, 20mg of chitosan was dissolved in 100 ml aqueous acidic solution to obtain the cation of chitosan. This aqueous solution was prepared with 80 ml of water, 15 ml of TPP and 5 ml of acetic acid. The solution was subjected to the constant magnetic stirring for 10 min and the pH of the solution was adjusted to 6.5. During the process involving chemical reaction, chitosan undergoes ionic gelation and precipitates to form spherical particles.

3.19.1. Characterization of chitosan Nanoparticles

Mean particle size and zeta potential of chitosan nanoparticles were determined by using Horiba scientific Nanoparticle Analyser SZ-100 series (Kyoto, Japan) at 25°C. Particle size analysis was performed by dynamic light scattering (DLS) method. For this, 1 ml of the nanoparticles suspension sample was taken in a cuvette. The cuvette was placed immediately inside the sample holder of the particle size analyser. Once the required intensity was reached, analysis was performed to obtain the mean particle size (n=3) and polydispersity index (PDI) of the sample.

Measurement of the zeta potential gives an idea about the stability of nanoparticles and it was determined based on laser doppler electrophoresis technique in the same instrument.

3.19.2.Nano-conjugation of the pcDNA4-StARorf with chitosan

Conjugation of nanoparticles with plasmid was carried out following the method given Nandanpawar et al., 2013 with slight modification. Briefly, 1 ml of chitosan nanoparticles solution having a concentration of 0.02% was dissolved in 4 ml triple distilled water making the volume upto 5ml. The plasmid solutions were prepared by diluting plasmid in concentration of 1μg/ul in nuclease free water. Chitosan and plasmid solutions were heated separately at 55^0C. Plasmid (1μg/ul) solution was mixed with nanoparticle (40mg/L) solution and vortexed at 2500 rpm for 30 seconds. The resulting mixture was stored at room temperature. On the next day, animals were treated with DNA nanoconjugates through intramuscular injection in cement tanks.

3.19.3 Confirmation of DNA conjugation with nanoparticles

Conjugation of DNA with the chitosan nanoparticle was confirmed by gel retardation assay. The stability of the nanoconjugated DNA were checked by incubating it with10 mg/ml DNAse I solution at 37^0C for about half an hour. The reaction was stopped by adding EDTA at 65^0C for 5 min. The integrity of the plasmid was analyzed by agarose gel electrophoresis (1.0% agarose).

3.20. *In vivo* Experiments

3.20.1 Experimental animals

The Asian catfish, *C. batrachus* were acquired from a private fish trader in Kaikaluru, Andhra Pradesh. The animals were reared in small earthen cement ponds. The animals were fed with commercial pelleted feed twice in a day. Water exchange was carried out in 2-3 days. Animals were acclimatized for 5-6 days prior to treatment.

3.20.2. Experimental Design

The *in vivo* studies were conducted on *C. batrachus* weighing 160-200gm. The animals were divided into 2 treatment groups and 2 control groups called Treatment 1 (TR1), Treatment 2 (TR2), Control 1 (CTL1) and Control 2 (CTL2).

On day 0 the animals in CTL 1 group were injected with 100µl blank chitosan nano-particles. CTL 2 with empty pCDNA4/HisMax A vector, TR1 with Chitosan-pcDNA4-StARorf conjugate and TR2 with pcDNA4-StARorf construct at the rate of 0.5µg/ g body weight in a constant volume of 100µl. Duplicates were run. Eight cement tanks of 10 animals each were included in treatment, and sampling was done on 12, 24, 48, 72 and 120 hours. Two animals were collected at random from all the tanks on each sampling time point. All injections were administered intramuscularly.

3.20.3. Collection of Tissues

The animals were decapitated in accordance with the Institutional Animal Care and Use Guidelines. Animals were anesthetized using clove oil and kept on ice before dissection. Sampling was done after 12, 24, 48, 72 and 120 hours of injection. During each sampling two-two individuals were taken out from each treatment group and blood was drawn directly from the caudal vein through the syringe and stored in 2 ml tubes. The tubes were kept for 2 hour at room temperature for clotting of the blood. After that the tubes were centrifuged at 5,000rpm for 20 min to pellet the cell debris. The cell free serum was removed and stored at -80^{o}C till further analysis. Further Testis, Liver, Kidney, Brain and Mussels were dissected out from the animals on ice aseptically and preserved in absolute alcohol for DNA isolation, NBF for histology and Qiagen’s RNAlater™ solution for RNA isolation. The samples for RNA isolation were stored in -80^{o}C till further analysis.

3.21 Nucleic acid isolation

3.21.1 Genomic DNA Isolation by Phenol-Chloroform Method

Genomic DNA was isolated from testis, brain, liver, kidney and muscle tissue of the *C. batrachus* following Sambrook *et al.* (2001). Composition of reagents is given in appendix I. Briefly the tissue was collected from the specimen using sterile scissors and forceps, taking care to avoid contamination. For DNA isolation, 100 mg of fresh tissue was taken in a 2.0 ml microfuge tube and chopped finely using sterile scissor. To each sample, 500 µl of DNA extraction buffer, 50 µl of 10% SDS and 5 µl of proteinase K was added and mixed well and incubated at 55°C for 16-18 hours in a shaker water bath for lysis. To the lysate, equal volume of

Tris-saturated phenol (Bangalore Genei) was added, mixed thoroughly until emulsified and centrifuged at 10,000 rpm for 10 min at 4°C. Aqueous supernatant was transferred carefully to a 2.0 ml microfuge tube and extracted with equal volume of phenol:chloroform:isoamyl alcohol mixture (25:24:1) and subsequently with equal volume of chloroform:isoamyl alcohol. After centrifugation the top aqueous layer containing DNA was transferred to a fresh 1.5 ml microfuge tube. The DNA was precipitated using 1/10th volume of 3M sodium acetate and 0.6 volumes of isopropanol. Following centrifugation at 10,000 rpm for 10 min at 4°C, the DNA was washed with chilled 70% ethanol, vacuum dried and resuspended in 30µl of TE buffer (pH 8.0). DNA was then incubated with 1 µl of RNase A at 37°C for 1 h to remove RNA and stored at -20°C for further use.

3.21.2. Total RNA isolation

RNA was isolated by Trizol method and quantified by Nanodrop.

3.21.3 DNase Treatment

A total 2.5µg RNA is used for DNase treatment from each sample following the method described in section 3.6.4.

3.21.4 cDNA synthesis

After DNase treatment, nanodrop reading was taken and 1µg DNase treated RNA was used form each sample for cDNA synthesis. Protocol is described in section 3.6.5.

3.22. Analysis of Gene Expression

3.22.1. Tissue distribution study

DNA was isolated from testis, brain, kidney, liver and muscles by using standard phenol chloroform method (Sambrook *et al.* 2001). Vector based primers pCMVF/R (Table 1) that amplified a 650bp region was used.

3.22.2. Gene expression by Real time PCR

Real time PCR was performed on Roche Light Cycler 480 thermocycler using 5x HOT FIREPol® EvaGreen® qPCR Mix Plus. The reactions were set in 25µl of total volume using 1µl of cDNA reverse transcribed from 1µg of total RNA, 0.5 ul of each forward and reverse pimers, 5 µl 5x HOT FIREPol® EvaGreen® qPCR Mix

Plus and nuclease free water to make up the volume. Each cDNA was prepared from pooled total RNA from 3 individuals at every time point and for all treatments. The PCR cycle consisted of an initial denaturation step at 94^{O}C for 15 min, followed by an amplification step (40 cycles of 95°C for 20 sec/60°C for 20 sec/72°C for 30 sec), a single melting curve step of 95°C for 10 sec/65°C for 1 min/97°C and finally a cooling step of 40°C for 10 sec.

3.22.3. Histology

For assessment of the effect of the treatment at tissue level, histology of tissues such as testis and liver was performed following the method given by Lightner (1997). The composition of reagents used has been given in Annexure I. The tissues for preparation of histological slides were dissected at 10th day after the injection aseptically and fixed using Neutral buffer formalin (NBF). One of the testes from the two males of each tank were fixed for histology. Thus there were 2 samples from every treatment group. In short, the fixed tissues were dehydrated in ascending grades of alcohol and embedded in paraffin wax to form blocks. Sections of 3µm thickness were cut from these blocks using a microtome and placed on a glass slide. The tissue sections were rehydrated using descending grades of alcohol and stained with eosin stain. The sections were then mounted with a cover slip and DPX mountant and observed under a light microscope. The slides were photographed using Carl Zeiss microscope camera.

3.23. Analysis of data

The raw Ct data from the real time experiments were converted into fold change values by the $\Delta\Delta$Ct method (Schmittgen and Livak, 2008). The fold change data was statistically analysed using ANOVA following the Duncan’s test using SPSS 16.0 software. Data on serum hormone levels was subjected to analysis of variance (ANOVA), followed by Duncan’s Multiple Range Test with the help of SPSS-16.0 version software. All the data analysis was expressed as mean ± standard error.

4. RESULTS

4.1. Isolation of total RNA

The total RNA isolated from the testis of *C. batrachus* was quantified using Nanodrop spectrophotometer (Thermo Fisher scientific, USA) and concentration was found to be 1400ng/µl. The ratio of absorbance at 260/ 280nm was 2.01, which indicated that the purity of the isolated RNA was good. The integrity of the extracted RNA was verified by running it on a 1% agarose gel, which showed clear separation of 28S and 18S rRNA bands (Fig.2).

4.2. Designing of primers

The Steroidogenic Acute Regulatory Protein (StAR) gene targeted in this study shares more than 70% homology with other vertebrates. The sequence was retrieved from NCBI (GenBank: KJ662668.1). Primers were designed to amplify full cds of 858bp sequence specific to the StAR gene (Table 1). The fragment amplified by these primers was cloned in-frame into pCDNA4/HisMax A vector under the control of CMV promoter.

4.3. PCR amplification of StAR gene

A full cds of StAR gene was amplified by using total RNA of *C. batrachus* testis by RT-PCR. The full ORF fragment of 858bp was amplified using the specific primer set (Fig. 3).

4.4. Cloning and screening of the recombinants

PCR product was extracted from the agarose gel and T/A cloned into pTZ57R/T vector. The DH5α strain of *E. coli* transformed with ligation mixture produced blue and white colonies in the X-gal/IPTG/ampicillin LB Agar. White colonies were selected as recombinant clones. The recombinant clones were confirmed by colony PCR using the gene specific primers (Fig.4). Plasmids were purified from positive colonies and checked on 1% agarose gel.

4.5. Construction of StAR gene expression plasmid

The StAR gene expression construct was developed in pcDNA4/His-Max A vector for *in vitro* and *in vivo* expression study. The schematic diagram of the construct has been shown in Fig.5. The recombinant were confirmed by restriction enzyme digestion (Fig.6). The clones were further confirmed by sequencing. The sequencing results were BLAST in NCBI that showed more than 99% similarity with the reported *C. batrachus* StAR gene. E value was found to be 0 and total score was 1531 out of 1531 which indicates that the full cds was cloned in the expression vector. The result of NCBI BLAST are shown in Fig 7.

4.6. *In vitro* expression of pCDNA4-StARorf

For *in vitro* expression pcDNA4-StARorf construct was transfected into SSN-1 cell line freshly sub-cultured 24 h prior to transfection. The cells were grown in 24 well cell culture plates. The transfected cells were harvested 48h post transfection and directly used for SDS-PAGE and western blotting. StAR is synthesized as a 30-kDa protein. The results of SDS-PAGE and western blotting are shown in Fig. 8.

4.7. Physicochemical characterization of Chitosan nanoparticles

The mean particle size of the chitosan nanoparticles was found to be 135.4 nm. The distribution of particle size is given in Fig. 9a and 9b. The nanoparticles were well dispersed and pretty stable as evinced by physico-chemical characteristics like polydispersity index (0.335) and zeta potential (17.7 mV).

4.7.1 Conjugation of chitosan nanoparticles with pcDNA4-StARorf

The plasmid was conjugated with chitosan nanoparticles and stability was checked by DNase treatment. After the treatment the conjugated plasmid was run on 1% agarose gel which shows the stability of conjugated plasmid (Fig. 10)

4.8. Tissue distribution of pcDNA4-StARorf

After 5 days of injecting the construct intramuscularly, the plasmid construct could be amplified from testis, brain, kidney and muscles (Fig 11). However, the plasmid was not detected from liver. Vector based primers pCMVF/R (Table 1) that amplified a 650bp region of the CMV promoter were used.

4.9. Expression profile of different reproductive genes

The expression profile of five major reproductive genes was studied by real time PCR. In the testis, expression of cyp11a1, cyp17a1, 3β-hsd, 17β-hsd and Aromatase (cyp19a1) genes were studied. Three housekeeping genes of *C.*

batrachus viz. β-actin, Elongase factor 1 (EF-1) and GAPDH were evaluated by melting curve analysis and Ct value. β-actin gene showed the lowest Ct value changes compared to other housekeeping genes and a specific melting peak. Hence this gene was used as the internal control (Fig. 12). The fold change in expression of the target genes in the treatment groups were estimated with respect to the control at each time point. The gene expression pattern of cyp11a1, cyp17a1, 3β-hsd, 17β-hsd and p450 aromatase (cyp19a1) in the experimental animals for the experimental duration has been depicted graphically in Fig.13.

In TR1 group the fold changes with respect to control groups was observed in all reproductive gene transcript. The cyp11a1 expression in TR1 did not show any significant change ($p < 0.05$) till 24h but increased up to ten folds on 5th day. The expression of cyp17a1 increased significantly ($p<0.05$) from day 3 and goes up to 6 fold on 5th day. The transcript level of 3β-hsd and 17β-hsd were also increased to 6.5 and 4 folds, respectively. There was no variation in expression pattern in the control group over the days of the experiment.

Unlike TR1, TR2 group did not show significant changes ($p < 0.05$) in all selected genes transcript throughout the experimental period. The expression of 3β-hsd and 17β-hsd increased two folds on day 1 and then stabilized at CTL 2 values on subsequent time points. The control group did not show any variation over the days of the experiment.

4.10 Serum hormone level

The treatment of fish with nanoconjugate (chitosan-pcDNA4-StARorf) showed significant effect on hormonal levels of 11-ketotestosterone (11-KT) at 72 and 120h and 17-β estradiol at 48 and 72h ($P<0.05$). The bar and line graph of 11-KT level is sown in Fig. 14 and 15. There was no significance difference observed up to 48h in TR1 group and TR2 group was not significantly different from control. Bar and line graph (Fig. 16 and 17) of 17-βE2 in TR1 group shows significant difference at 48h and 72h only not any other time point. TR2 group did not show any significant changes during entire experimental period.

4.11. Histological study of gonads

In the TR1 group, the histological appearance of testis was slightly different from the control on 10th day (Plate.1 A, B). The primary spermatogonial cells increases on the wall of seminiferous tubules and the number of spermatozoa also increases on 10th day whereas in TR2 group no significant changes in testis were observed compared to control group.

The histological examination of the liver tissue appearing to be normal with well-defined hepatic cells. The nucleus is clearly visible with its cellular margins in both treatment and control group at 10th day (Plate.2 A, B).

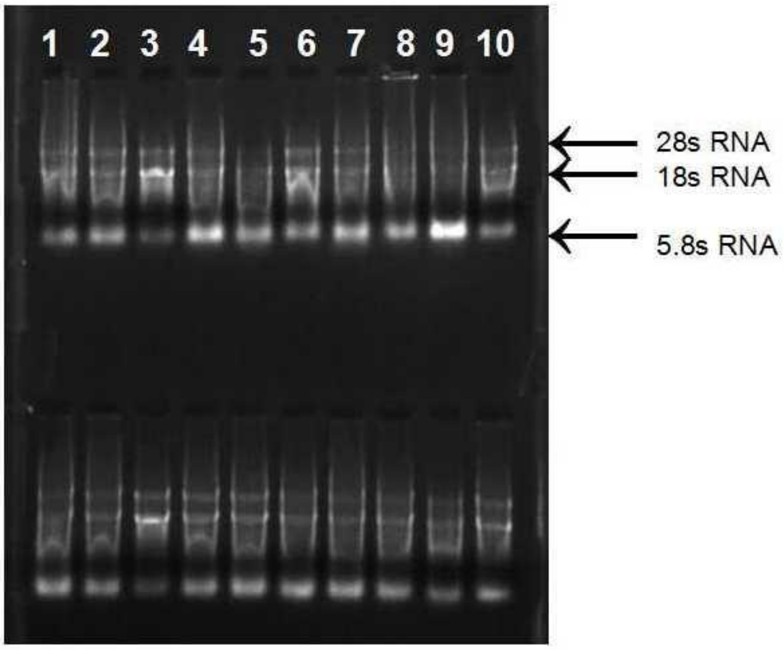


Fig. 2 RNA run on 1% agarose gel showing 3 different bands 28s, 18s and 5.8s RNA.

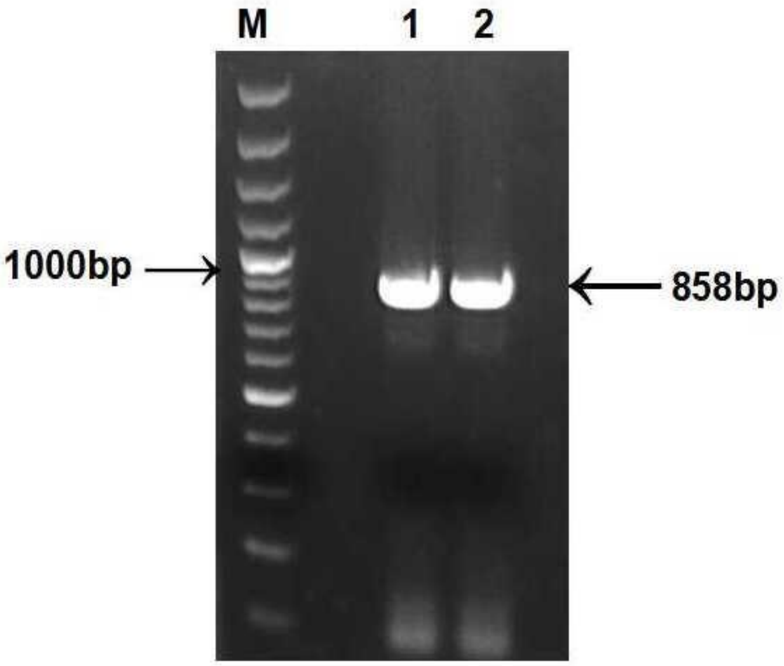


Fig. 3 PCR amplification of StAR ORF of amplicon size 858bp. Lane M; 100bp plus ladder (Thermo Fisher scientific USA), lane 1-2: Amplified products at 58°C annealing temperature.

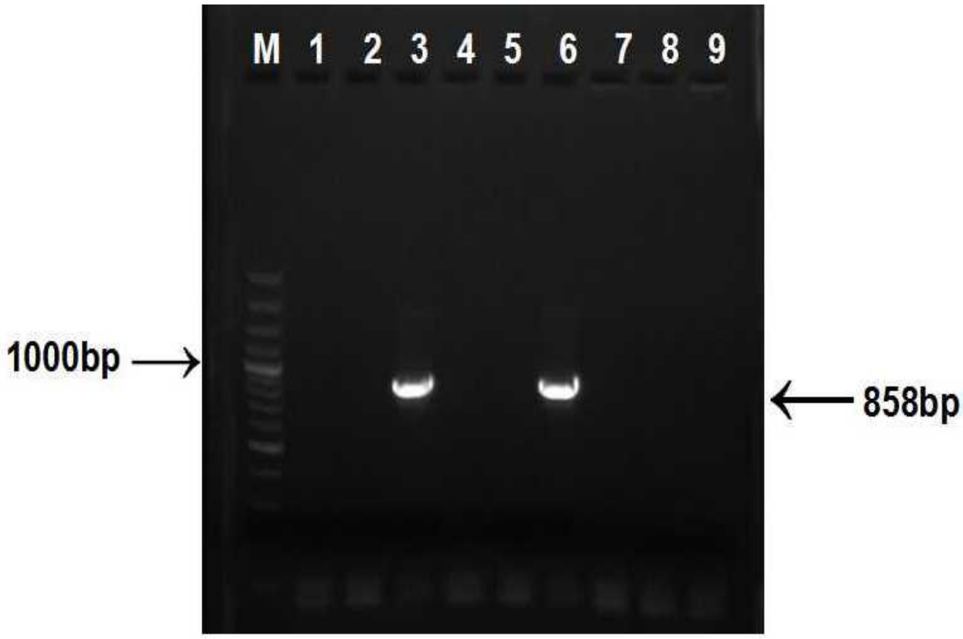


Fig. 4 Colony PCR of StAR fragment from recombinant clones. Lane M; 100bp plus ladder (Thermo Fisher scientific, USA); Lane 3 and 6 showing amplified product of StAR gene from positive colonies.

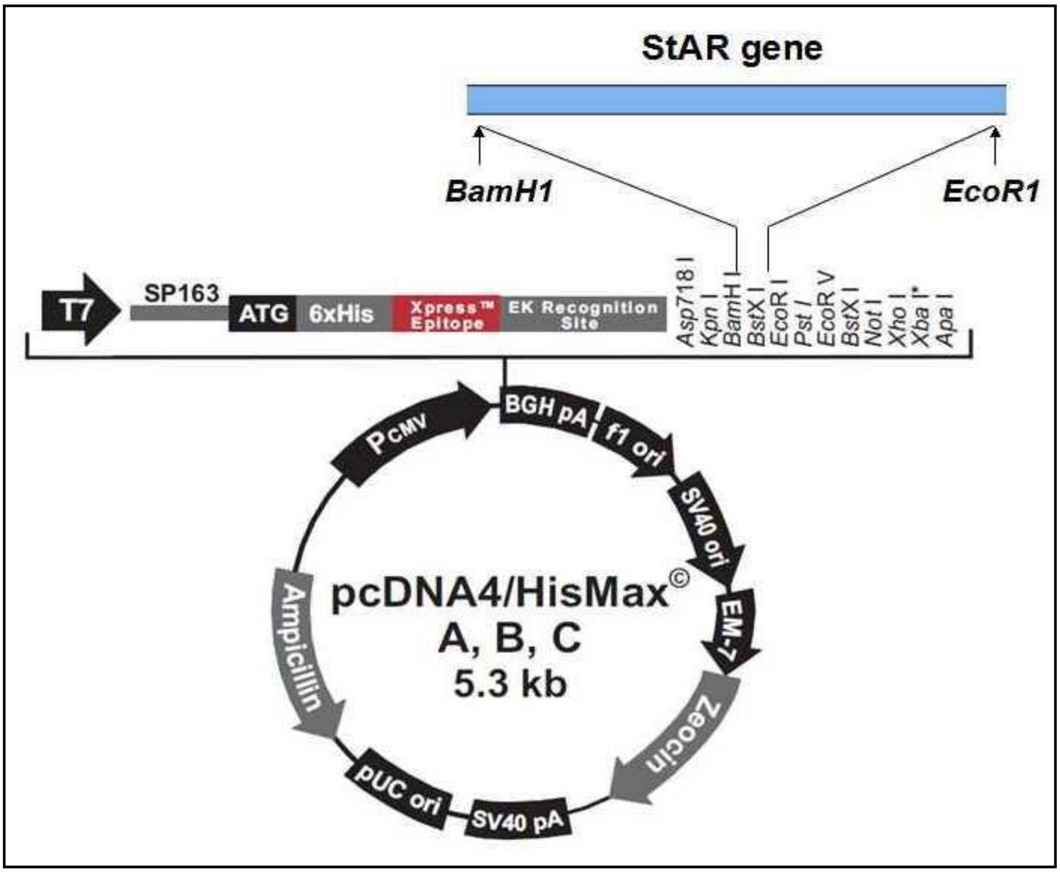


Fig. 5 pcDNA4-StARorf construct designed for *in vivo* expression.

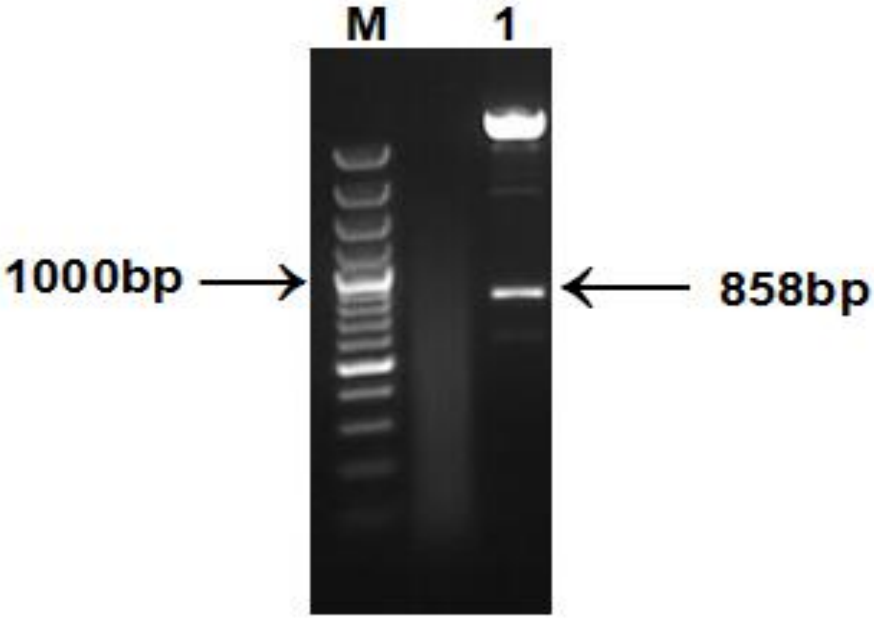
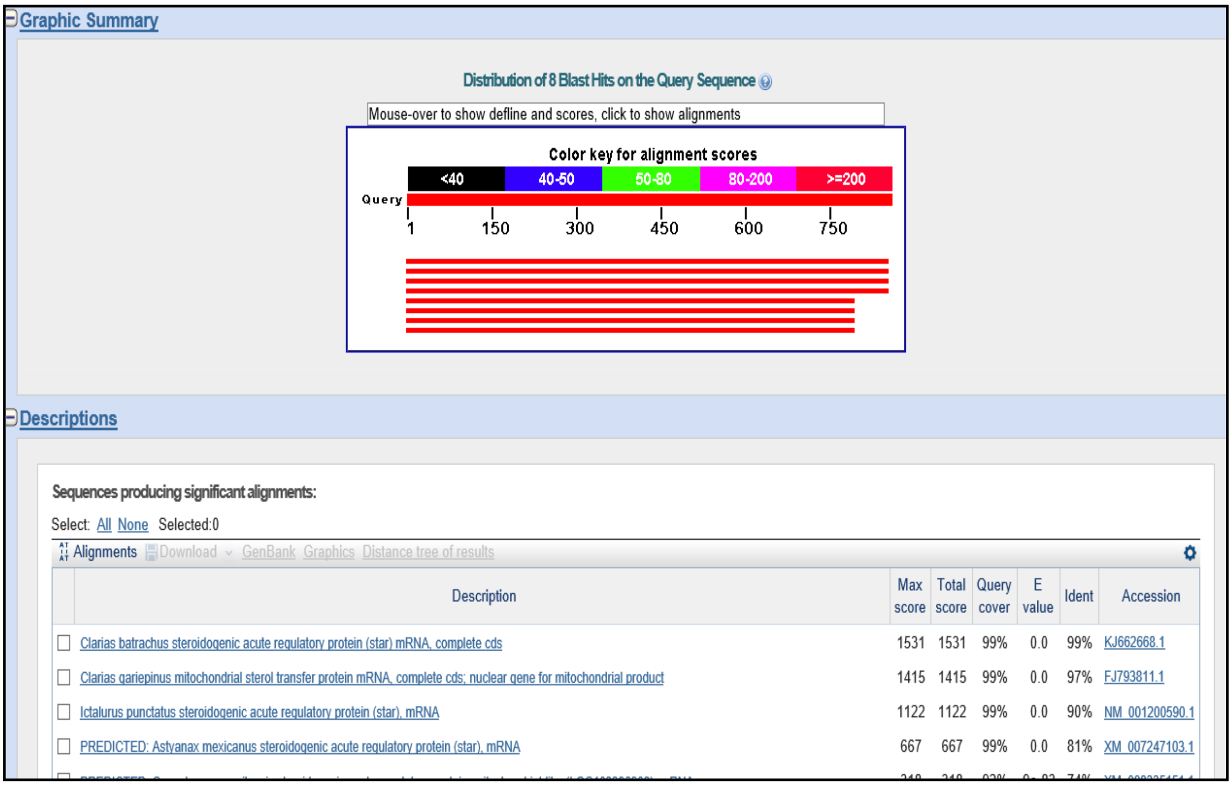


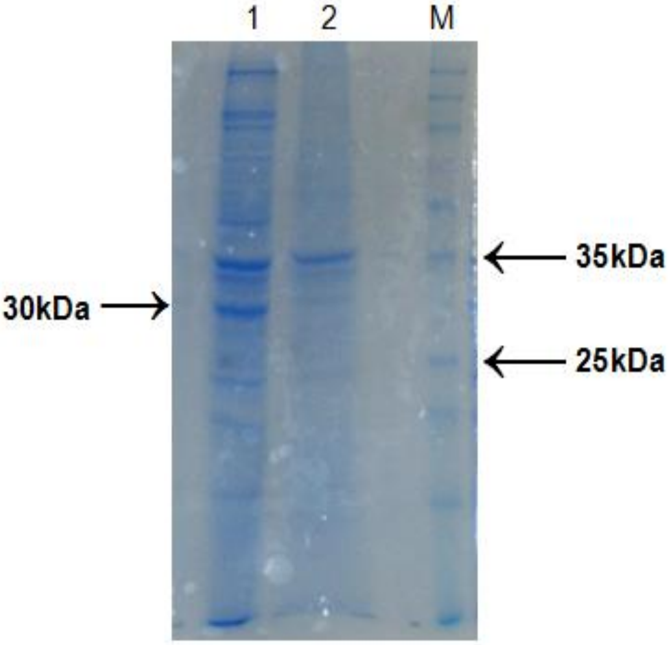
Fig. 6 Restriction enzyme digestion of pcDNA4-StARorf construct. Lane M; 100bp plus ladder (Thermo Fisher scientific USA), lane 1: R.E digestion of pcDNA4-StARorf with *BamH1* and *EcoR1* releases insert of size 858 bp.



Sequences producing significant alignments:

	Description	Max score	Total score	Query cover	E value	Ident	Accession
☐	Clarias batrachus steroidogenic acute regulatory protein (star) mRNA, complete cds	1531	1531	99%	0.0	99%	KJ662668.1
☐	Clarias gariepinus mitochondrial sterol transfer protein mRNA, complete cds; nuclear gene for mitochondrial product	1415	1415	99%	0.0	97%	FJ793811.1
☐	Ictalurus punctatus steroidogenic acute regulatory protein (star), mRNA	1122	1122	99%	0.0	90%	NM_001200590.1
☐	PREDICTED: Astyanax mexicanus steroidogenic acute regulatory protein (star), mRNA	667	667	99%	0.0	81%	XM_007247103.1

Fig.7 BLASTn analysis of sequencing result



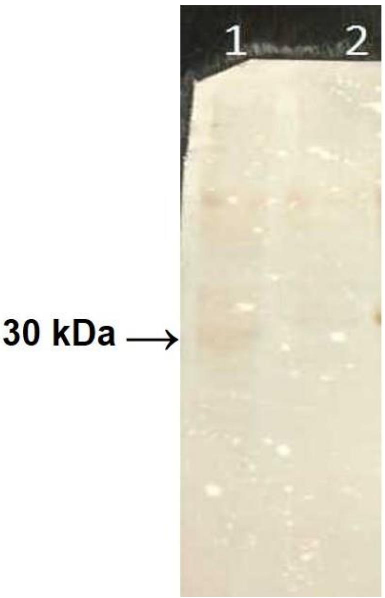


Fig. 8a SDS-PAGE of cells transfected with pcDNA4-StARorf, Lane M- Abcam Prism Ultra Protein Ladder (10 - 180 kDa); Lane 1 sample and 2 control.

Fig. 8b Western blotting; Lane 1 sample and 2 control.

\

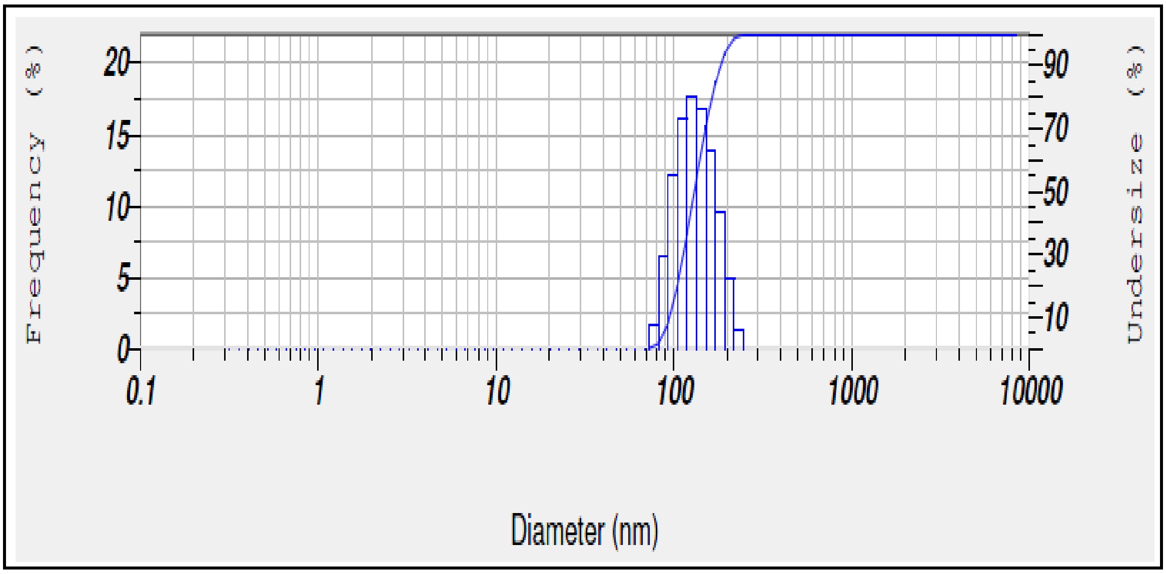


Fig. 9a Mean diameter of chitosan nanoparticles.

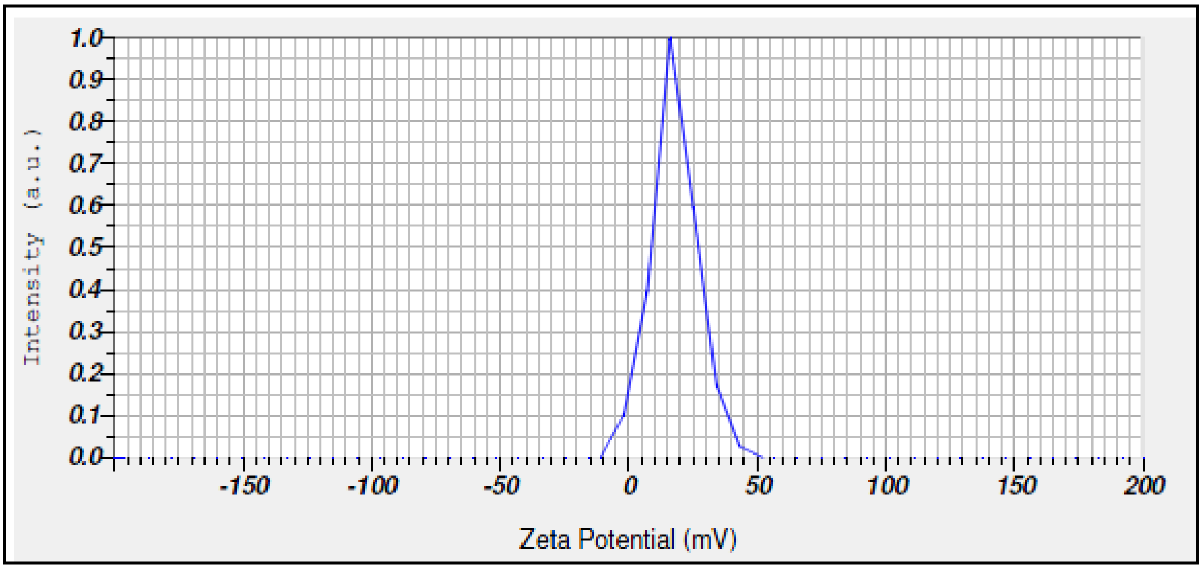


Fig. 9b Zeta potential of chitosan nanoparticles.

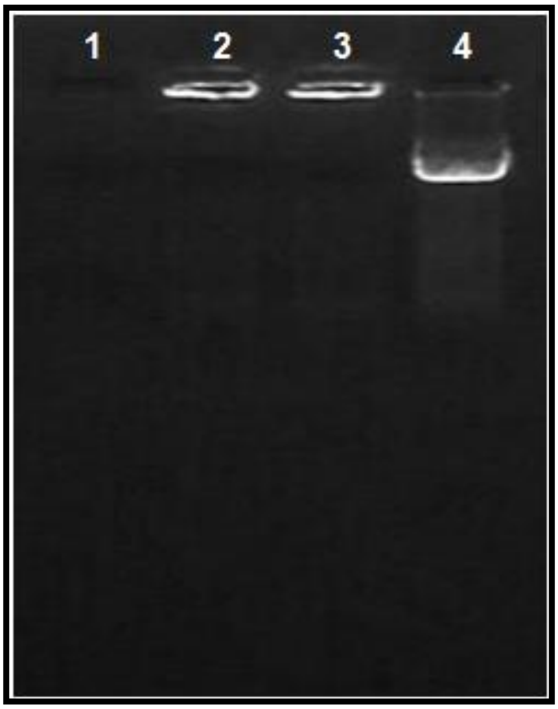


Fig. 10 Gel retardation assay Lane 1; Naked plasmid digested with DNase, 2; Chitosan conjugated plasmid digested with DNase, 3; Chitosan conjugated plasmid, 4; Naked plasmid

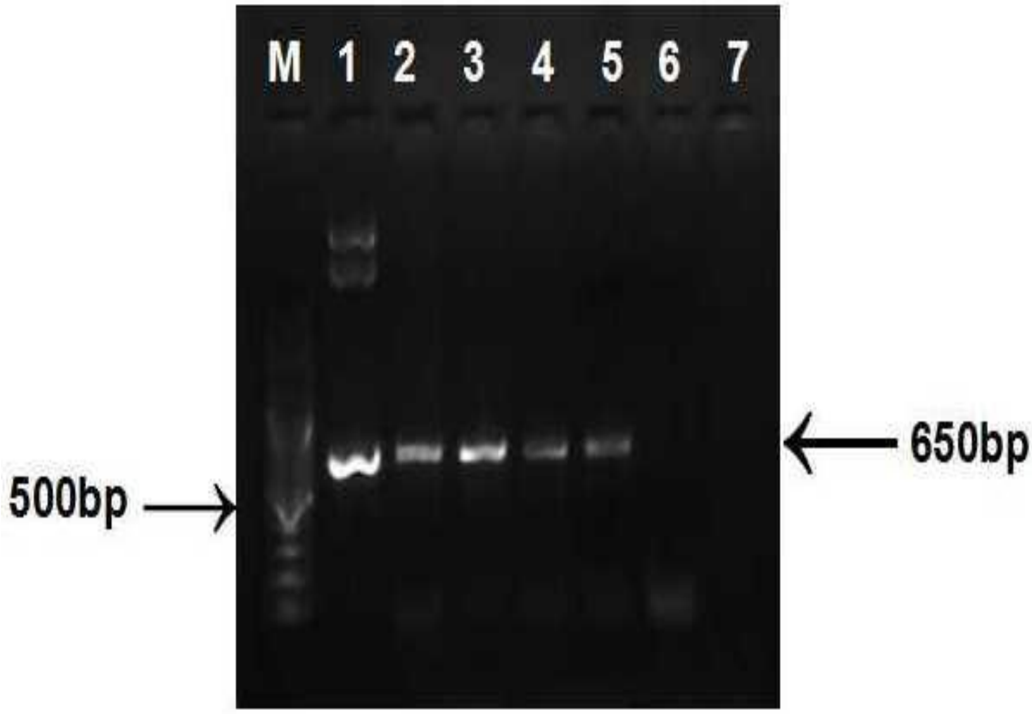


Fig. 11 Tissue distribution of pcDNA4-StARorf in 5 different tissues of *C. batrachus* as determined PCR. Lane M; 100bp plus ladder (Thermo Fisher scientific, USA); lane 1 positive control; 2 Testis, 3 Muscle, 4 Brain, 5 Kidney, 6 Liver and 7 negative control.

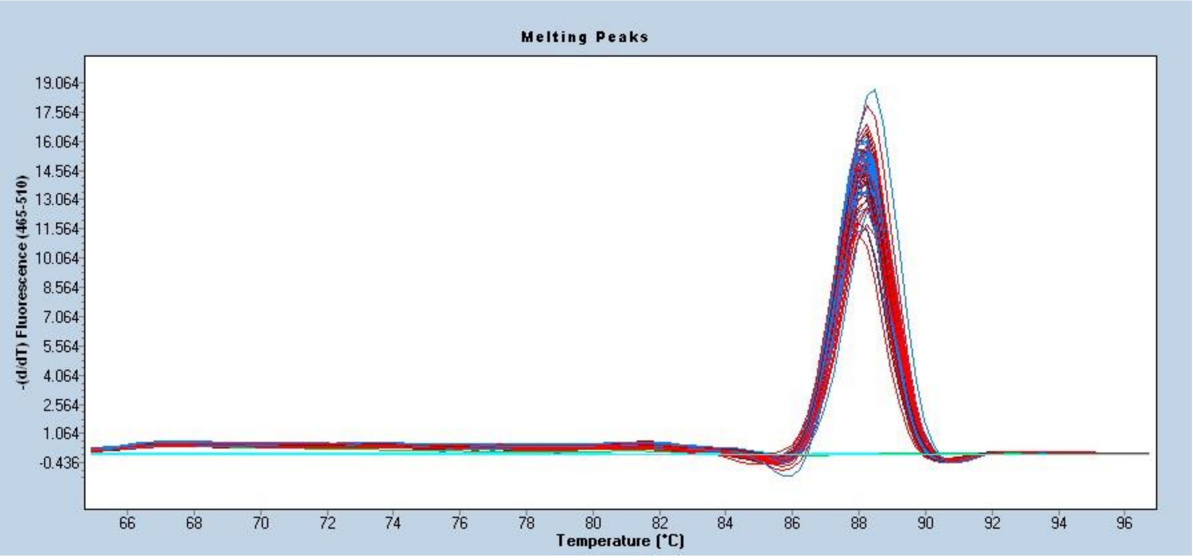


Fig.12.1 Melting curve of beta-actin- housekeeping gene

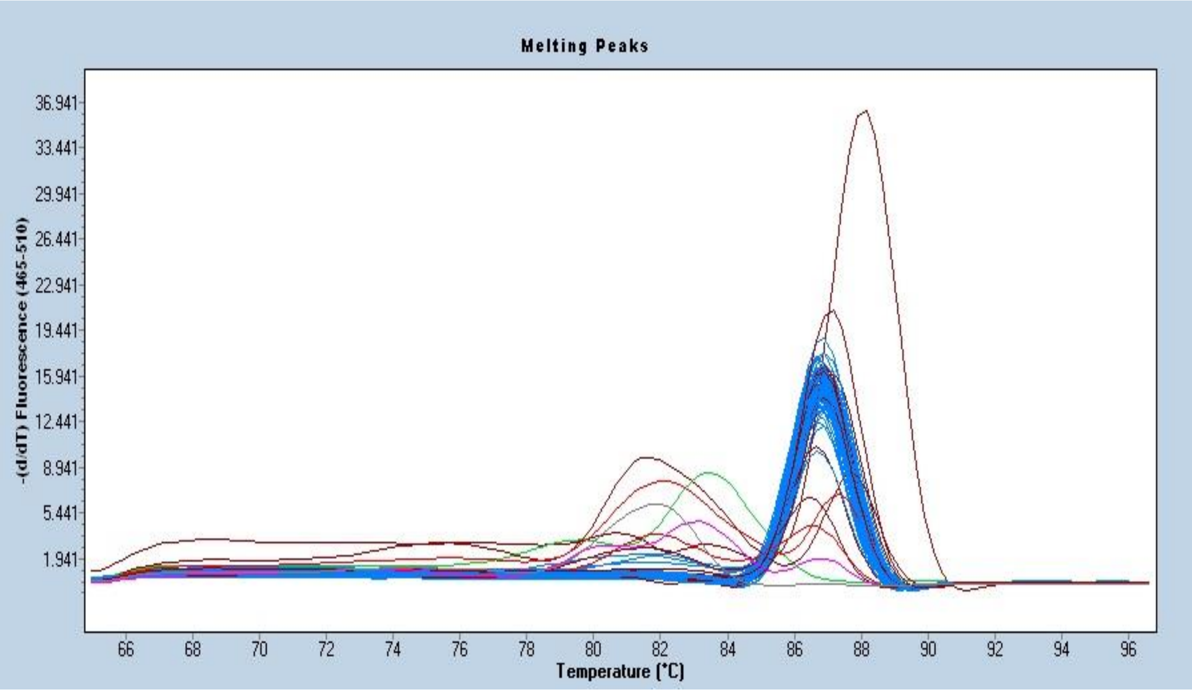


Fig.12.2 Melting curve of Elongase factor (EF)- housekeeping gene

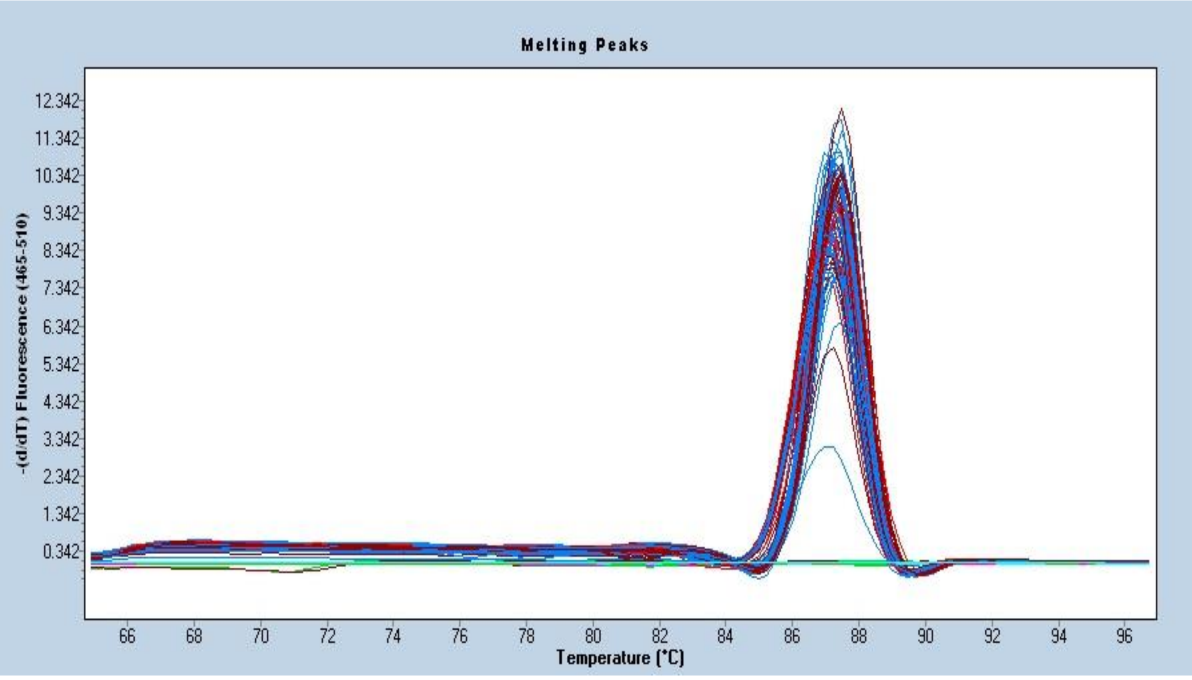


Fig.12.3 Melting curve of GAPDH- housekeeping gene

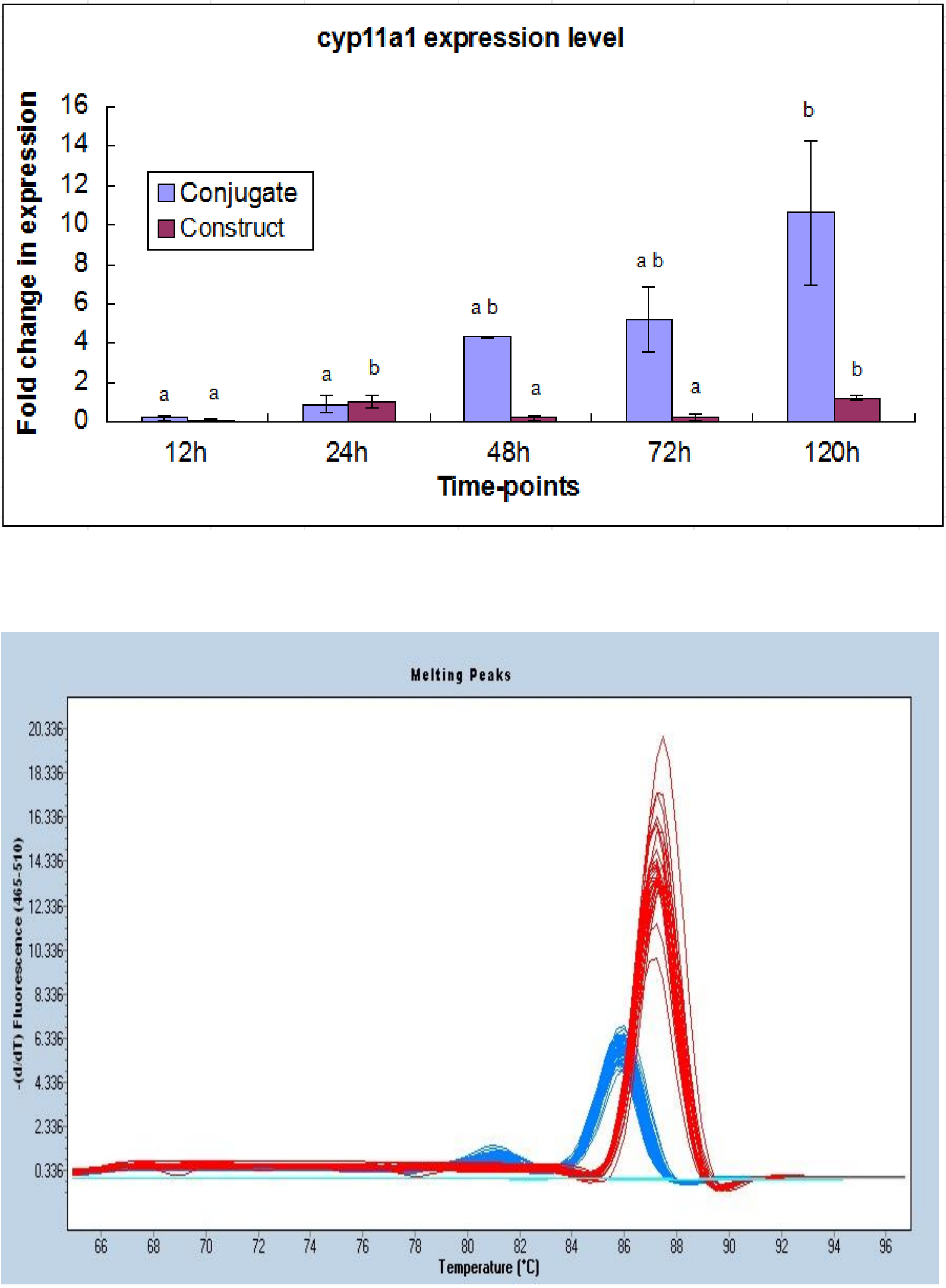


Fig.13.1 Fold change in expression and melting curve of cyp11a1 gene after the delivery of construct with respect to control at different time points.

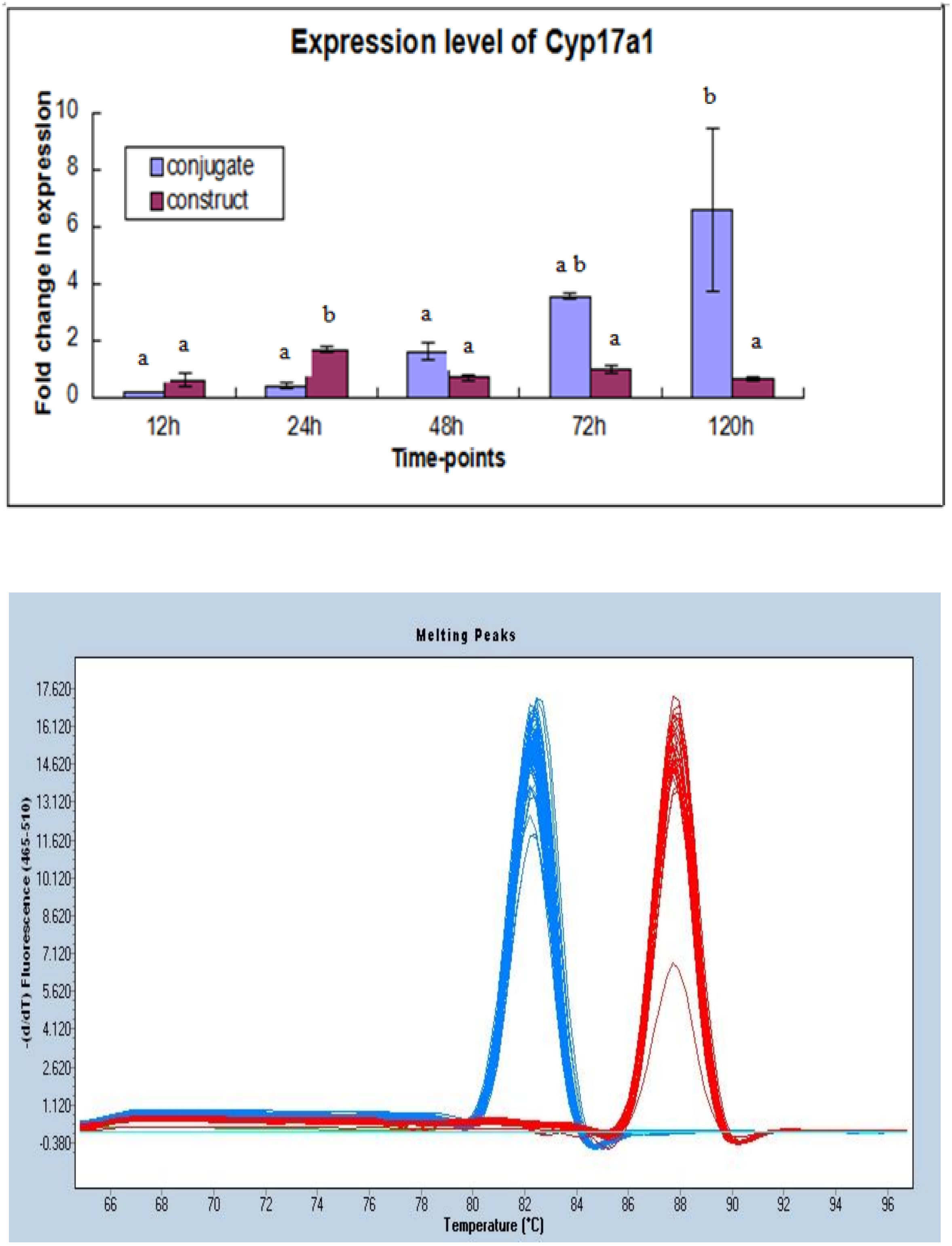


Fig.13.2 Fold change in expression and melting curve of cyp17a1 gene after the delivery of construct with respect to control at different time points.

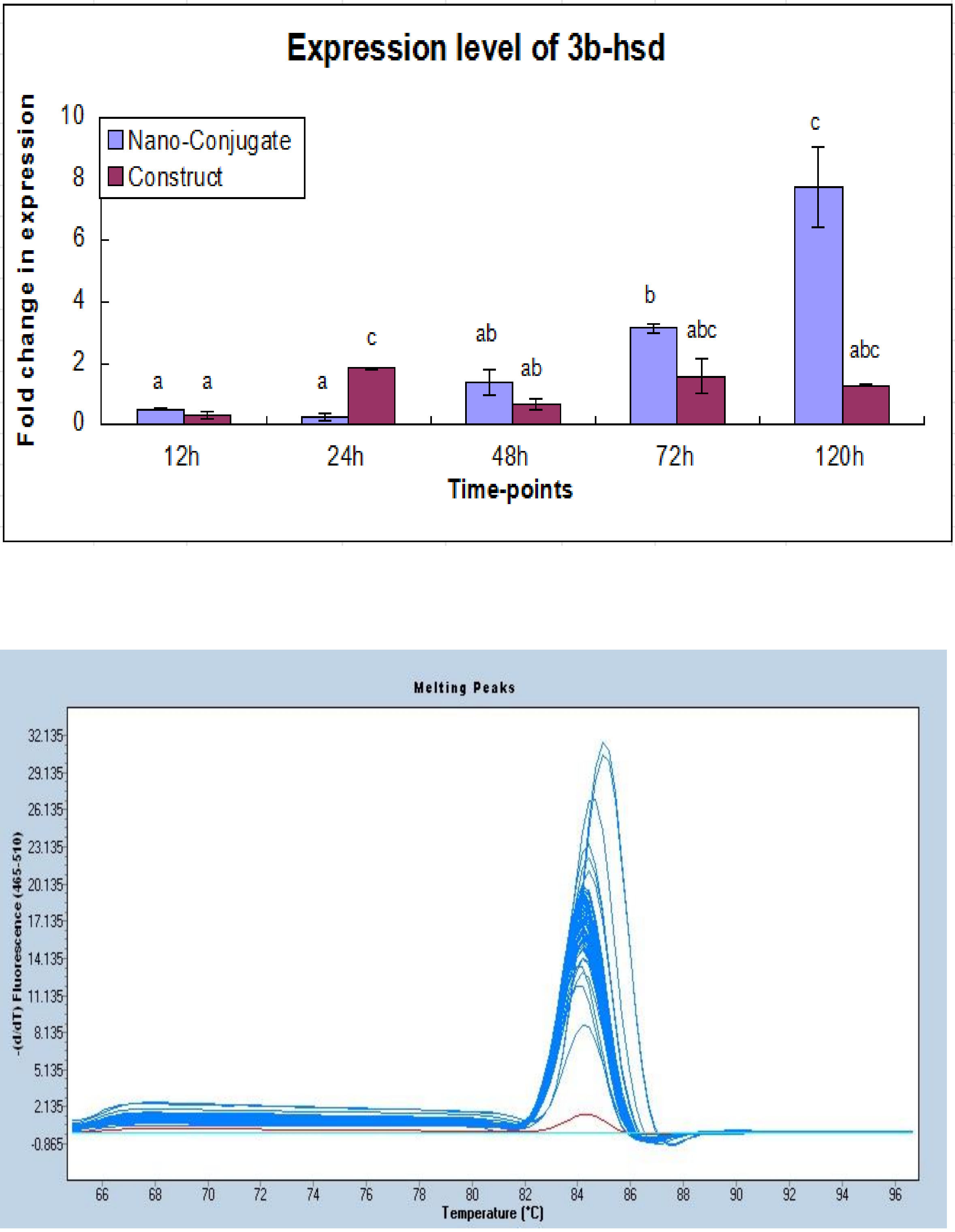


Fig.13.3 Fold change in expression and melting curve of 3-β-HSD gene after the delivery of construct with respect to control at different time points.

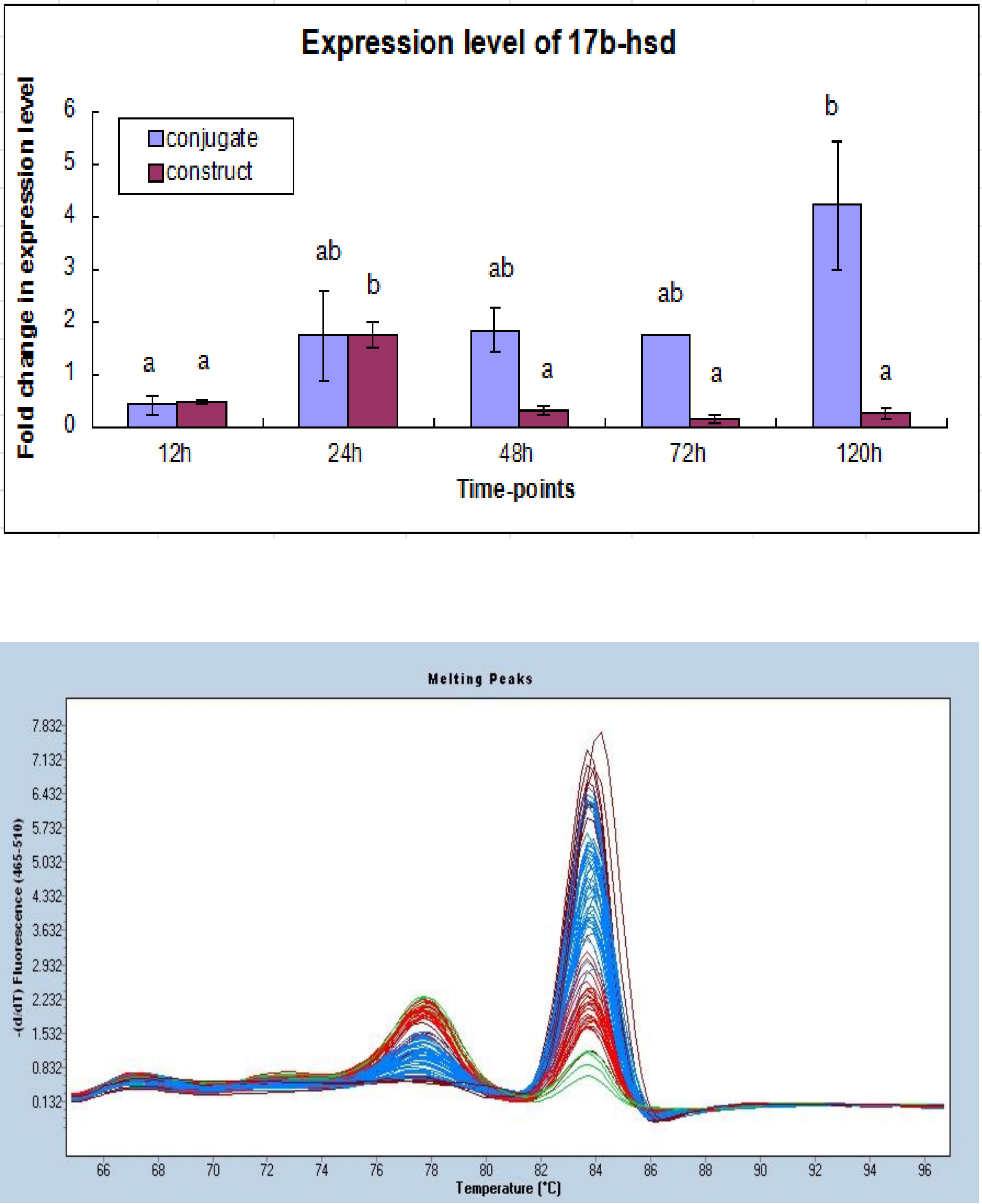


Fig.13.4 Fold change in expression and melting curve of 17-β-HSD gene after the delivery of construct with respect to control at different time points.

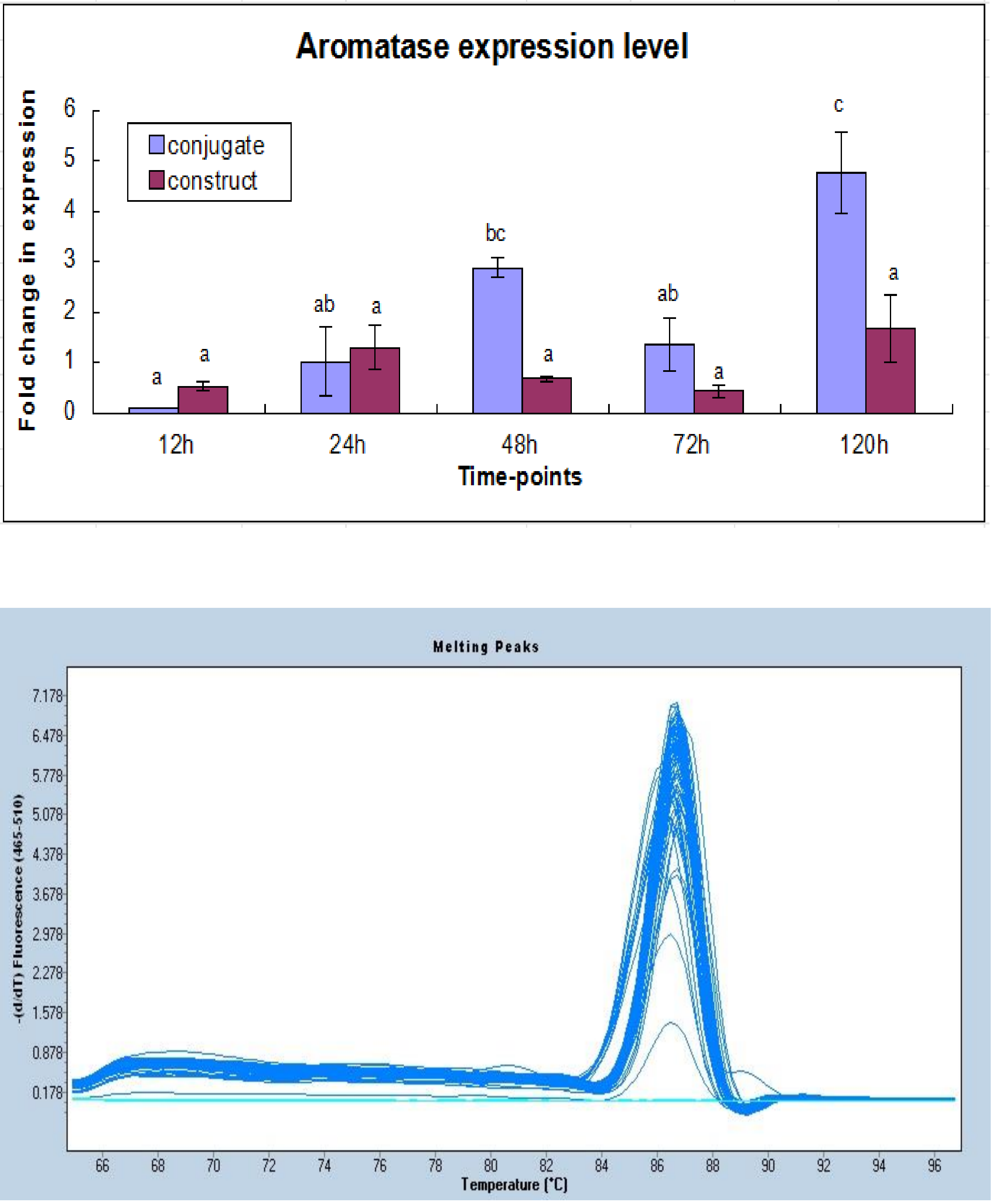


Fig.13.5 Fold change in expression and melting curve of P450 aromatase gene after the delivery of construct with respect to control at different time points.

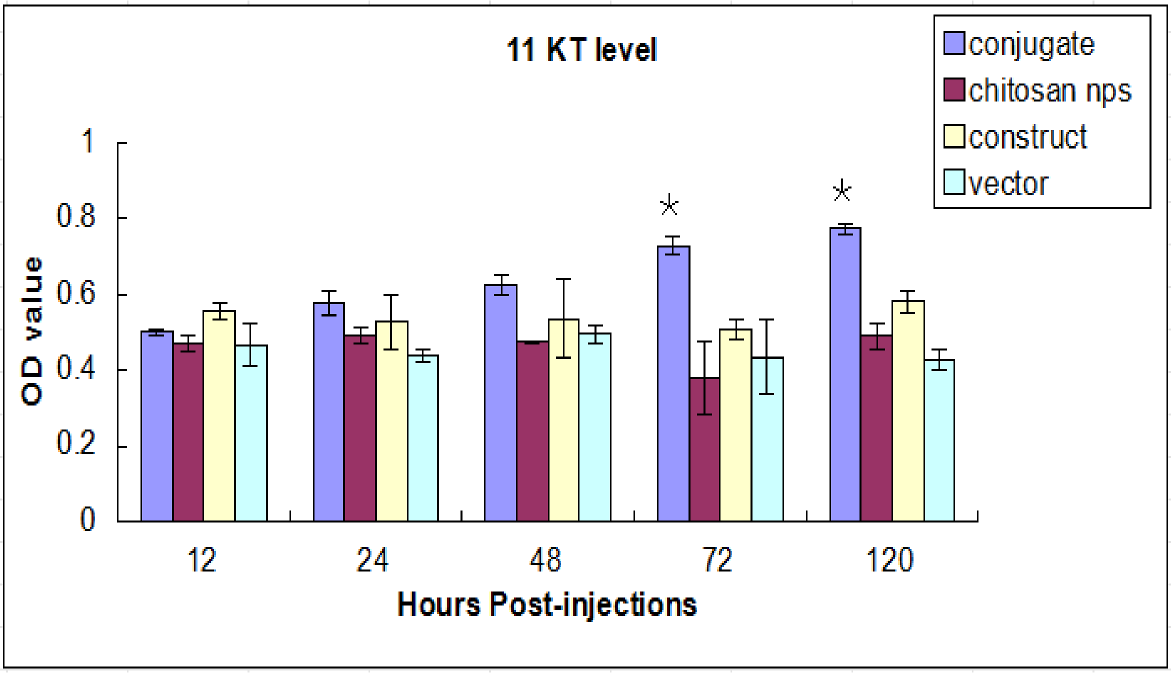


Fig. 14 Time-points versus OD value bar graph of 11-ketotestosterone. Star sign indicate the significance at ($P<0.05$).

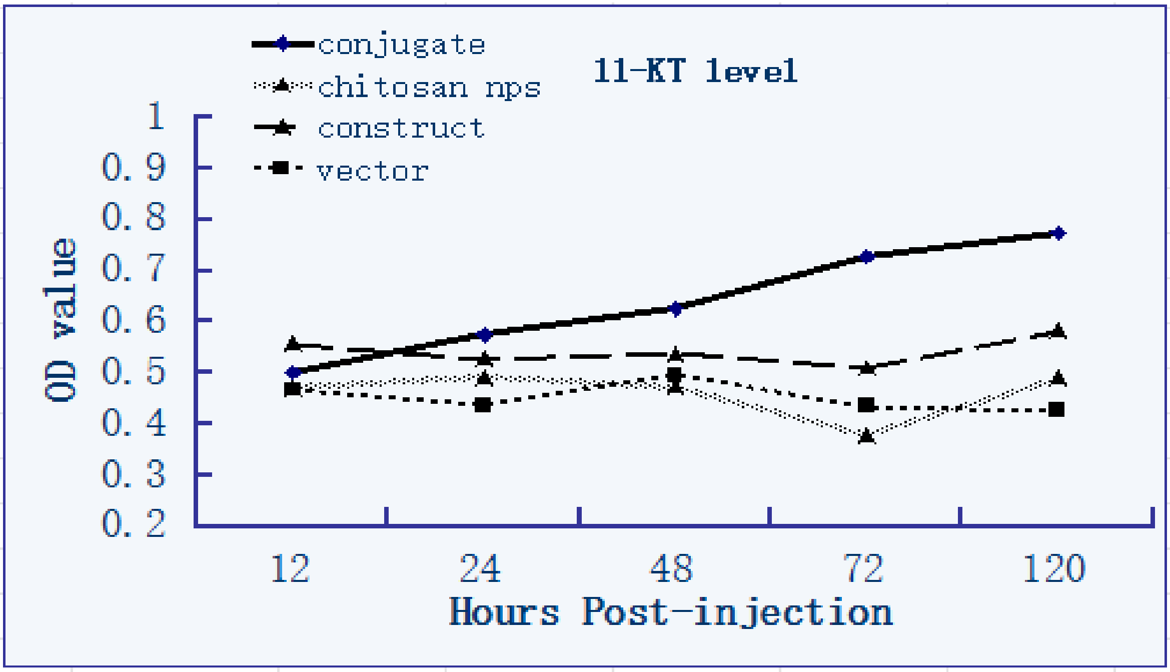


Fig. 15 Time-points versus OD value line graph of 11-ketotestosterone.

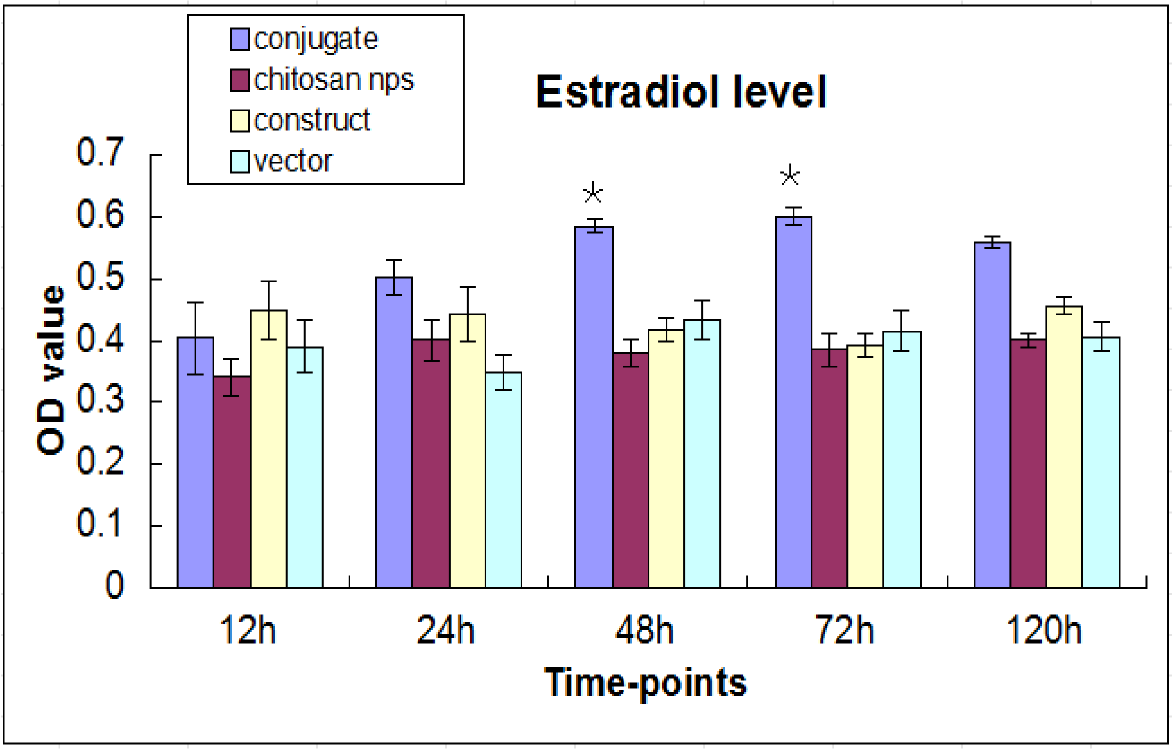


Fig. 16 Time-points versus OD value bar graph of estradiol. Star sign indicate the significance at ($P<0.05$).

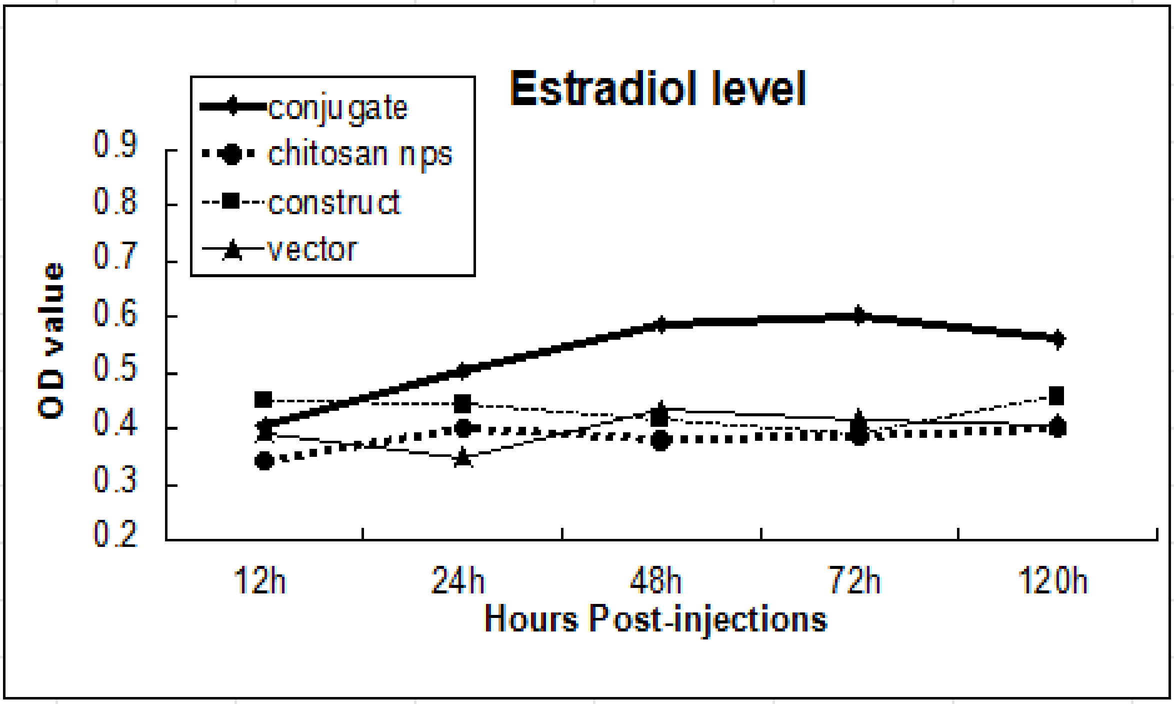


Fig. 17 Time-points versus OD value line graph of estradiol.

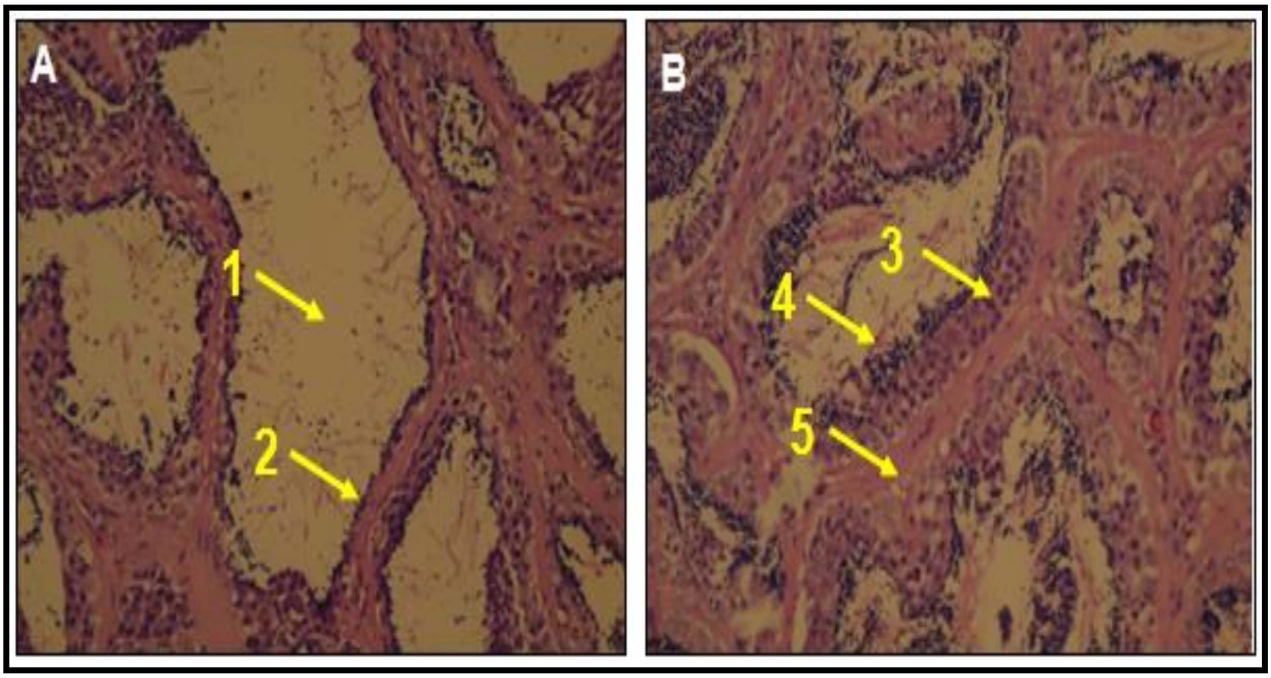


PLATE- 1. Testis tissue sections of *C. batrachus*
A) Control group showing empty lumen (1) and primary spermatogonial cells (2)
B) nano-conjugated plasmid exhibiting primary spermatogonia (3), spermatozoa (4) and leydig cells (5). HandE, 40X.

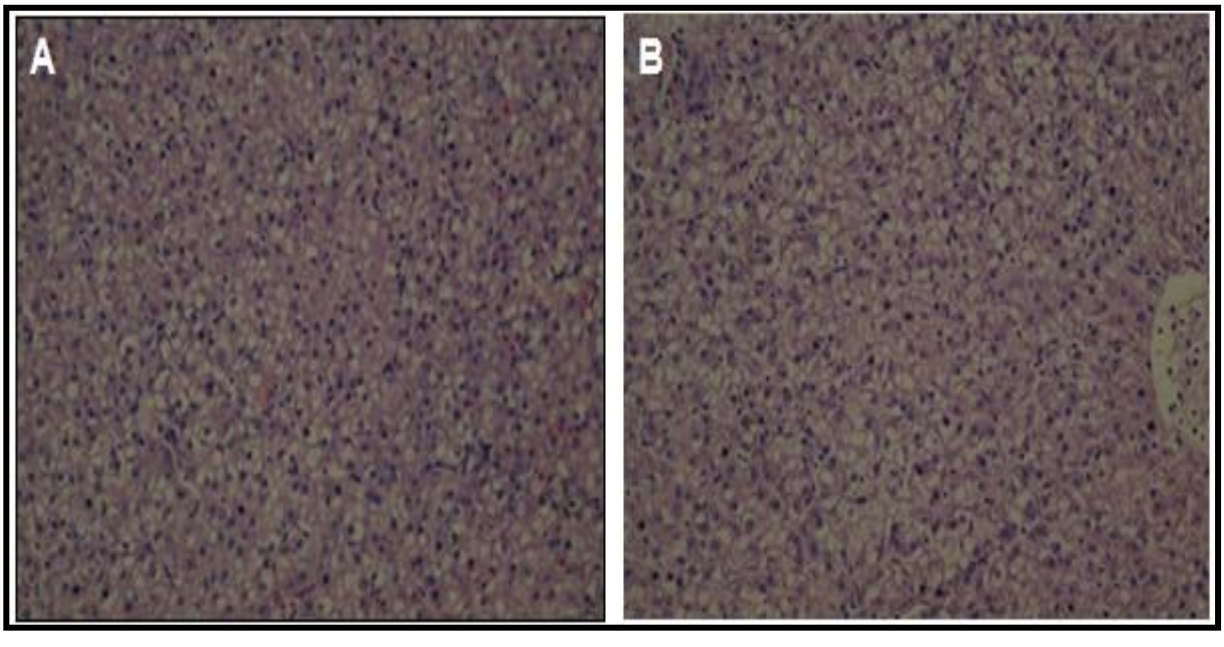


PLATE 2. Liver tissue section of *C. batrachus* appearing to be normal with well-defined hepatic cells. The nucleus is clearly visible with its cellular margins in both control (A) and treatment (B) group at 10th day. HandE, 40X.

5. Discussion

Aquaculture of C*larias batrachus* is constrained by non-availability of seed with natural collections diminishing considerably. Its complex reproductive behavior make induced breeding a complicated and requiring sacrificing of the male. The present work is carried out by focusing on current problem in *C. batrachus* reproduction. Steroid hormones play a crucial role in regulation of growth, development, differentiation, reproduction and several other functions in vertebrates (Sreenivasulu *et al.,* 2009). Steroidogenesis pathway of many catfishes was studied to understand the role of different genes (Kumar *et al.*, 2000; Sridevi *et al.*, 2013). Cholesterol plays a key role in synthesis of steroid hormones and involves different genes (Payne and Hales, 2004). The cholesterol is converted in to pregnenolone by the action of P450 side-chain cleavage enzyme in mitochondrial inner membrane. However, cholesterol cannot cross the aqueous layer between the outer and inner mitochondria membranes from cytoplasm and is delivered by a sterol transfer protein, steroidogenic acute regulatory protein (StAR) (Stocco *et al*., 2000). The true rate-limiting step in steroidogenesis is the delivery of cholesterol across mitochondrial membrane (Nematollahi *et al.*, 2012). Researchers reported that the StAR deficiency in humans will lead to congenital lipoid adrenal hyperplasia (lipoid CAH), a condition in which cholesterol and cholesterol esters accumulate and the new born fails to synthesize adequate levels of steroid hormones (Lin *et al*., 1995). This is further evidenced by StAR knockout mice which showed phenotypic mirrors of human lipoid CAH (Caron *et al*., 1997). Ruane *et al*. (2005) described the first case of congenital adrenal hyperplasia in common carp with low production of cortisol and enlarged head kidney.

The present study has focussed on the development of a nano-conjugated StAR gene expression construct which can trigger the steroidogenesis pathway in *C. batrachus*. For that a plasmid DNA construct (pcDNA4-StARorf) was designed for the expression of StAR gene *in vivo*. This construct was conjugated with chitosan nanoparticles for effective delivery. The effect of nano-conjugated StAR gene construct on selected reproductive genes such as Cyp11a1, Cyp17a1, 3β-hydroxysteroid dehydrogenase (3β-HSD), 17β-hydroxysteroid dehydrogenase (17β-HSD) and Cytochrome P450 aromatase was studied. Though StAR gene has been characterized in many fish species like zebrafish, rainbow trout, eel, cod and *Clarias gariepinus* (Bauer *et al*., 2000; Kusakabe *et al*., 2002; Li *et al*., 2003; Goetz *et al*., 2004; Sreenivasulu *et al.*, 2009b). However there are no reports on development of construct of the StAR gene in fishes.

The functionality of StAR gene construct was confirmed by transfecting the SSN-1 cell lines. The SDS-PAGE and western blot analysis with Anti His tag antibody shows that a recombinant protein of 30 kDa was form inside the cells. Yang *et al.* (2007) designed a recombinant plasmid construct by cloning *Lgeionella pneumophila* pilE gene in pcDNA3.1 (+) expression vector. They transfected the construct (pcDNA3.1-pilE) in to NIH3T3 cells that resulted in a strong green fluorescence on the cell membrane and inside the cell. A recombinant protein of 15.7 kDa was detected in the transfected cells with western blotting. In similar study, Das *et al*. (2015) find the pcD-GIH-lh construct effectively knocked down gih transcript both in vitro and in vivo in P*. monodon*. Rajeshkumar *et al.,* 2009 had made the chitosan conjugated VP28 gene construct in pcDNA3.1 expression vector and confirm the expression in SISK cell lines by fluorescence microscopy using primary antibody raised against VP28 of WSSV.

Nanocarrier-based vaccines have received much attention for effective immunization through better targeting and by triggering antibody response at the cellular level (Ramya *et al.*, 2014). Chitosan NPs are the most commonly used in the delivery of the biomolecules (Rather *et al*., 2013; Sharma *et al*., 2014). As chitosan carries positive charge and DNA has negative charge, they form a stable nanoconjugated DNA particle and do not get degraded by cellular enzymes such as nucleases present in the cell (Saranya *et al*. 2011). In this study, the plasmid was conjugated with chitosan nanoparticles for targeted and effective delivery of the construct. The nanoconjugated plasmid showed high level of expression of selected reproductive genes compared to naked plasmid. Similar results on effectiveness of nanoconjugated DNA has been reported by various researchers (Kumar *et al*., 2008; Rajeshkumar *et al*., 2009; Pathan *et al.*, 2013).

The nano-conjugated StAR gene construct was delivered intramuscularly in the experimental animals with a dose of 0.5µg/ gm body weight of fish in constant volume of 100µl. The intramuscular injection at this dose showed higher level of expression of all the selected genes and hormonal titre at 5th day compared to other time points. Romøren *et al*. (2004) reported that rainbow trout of 5gm body weight injected with 2 µg of plasmid intramuscularly (i.m.) showed 6.8 fold increase in luciferase expression while Rainbow trout of 60 gm showed 4.1 fold increase in luciferase expression when injected with 5 µg of plasmid intramuscularly (i.m.) on 5th day. There are very few reports available on the delivery of StAR gene construct to see its effect on reproductive genes. In a study Nunez *et al.* 2006 has reported that green monkey kidney cells (COS-1) co-transfected with Atlantic croaker StAR gene and human cholesterol side chain cleavage enzyme (p450ssc) expression constructs are able to produce significantly more pregnenolone than cells transfected with p450ssc alone. That shows that p450ssc enzyme is work only after the cholesterol delivery by StAR gene to the inner mitochondrial membrane. In another study Sugawara *et al*. 1995 found that the co-delivery of StAR and p450ssc in human COS-1 cells increased the pregelonone level more than 4 folds compared to control.

PCR amplification of vector based CMV promoter from brain, gonads, head, kidney and muscles showed that the plasmid is transported from its site of injection to different tissues. From liver, the plasmid could not be amplified, which showed that the plasmid is not retained in the liver. Manna *et al.* (2003) showed that regulation of steroidogenesis occurs in a tissue-specific manner and involves multiple signaling pathways, including protein kinase A (PKA) and protein kinase C (PKC), among others, this appears to be conserved across most vertebrate species. Sreenivasulu *et al.* (2009b) reported the predominant expression of StAR in gonads and liver of *C. gariepinus* by RT-PCR. In addition, transcript was also found in kidney, brain and intestine. Nunez *et al.* 2006 characterize the StAR gene of Atlantic croaker and tissue distribution study shows the StAR gene is expression only from head kidney and gonads not from brain. Thus the StAR gene expression is varies species to species but it predominantly expressed in steroidogenic tissues like testis, ovary and head kidney.

The expression level of selected genes at different time points after the delivery of nano-conjugated and naked plasmid DNA was analyzed. The expression in TR1 group increased continuously upto 5th day but in TR2 group the expression of all the genes increased first and then subsequently reached to the basal level on 5th day. This indicates that the nano-conjugated plasmid is more stable than the naked plasmid inside the cells. The expression of CYP11A1 gene increased to 10 fold on 5th day after injection of the nanoconjugated plasmid. The CYP11A1 catalyzes the first step in steroidogenesis, conversion of cholesterol into pregnenolone, and controls the synthesis rate of all steroid hormones (Richards *et al.*, 2013). The expression of other cytochrome family genes like CYP17A1 and CYP19A1 increased 6.5 and 4.7 folds, respectively at 5th day. CYP17A1 is a steroidogenic enzyme essential for the production of sex steroids in gonadal tissues and it possesses both the 17a-hydroxylase and 17, 20-lyase activities (Hinfray *et al.*, 2011). In all vertebrates, cytochrome P450 aromatase (Cyp19a1) converts C19 androgens into C18 estrogens, important hormones involved in the control of many important physiological processes, notably reproduction (Brion *et al.*, 2012). The expression of 3β-HSD gene and 17β-HSD gene increased up to 7.7 and 4.2 fold, respectively when injected with nano-conjugated construct with respect to control at 5th day. In all genes, the expression was maximum on 5th day and minimum at 12h which shows that the expression increased with time.

Sex steroid hormones play important roles at all stages of the reproductive cycle in vertebrates. Several studies on their structures and roles in mediating various stages of gonadal development and maturation in bony fishes have been carried out (Kime, 1993; Yaron, 1995). Application of certain steroids, particularly androgens, can induce precocious spermiation in the goldfish, *Carassius auratus* (Yamazaki and Donaldson, 1969; Crim *et al.*, 1976). In the present study, the level of 11-ketotestosterone showed increasing trend after 72 hours of nano-conjugated construct delivery. Whereas the level of estradiol increased initially but there was no significant difference ($p<0.05$) found at 5th day. From the above study we can say that the delivery of StAR gene increases the level of reproductive hormones which establishes the role of StAR gene in regulating reproduction in the fishes.

The effect of StAR gene administration on gonadal development was checked in testis in *C. batrachus*. After 10 days of nano-conjugated construct delivery, the more number of spermatozoa was found in seminiferous tubules which was not seen in the control group. This showed the effe ctiveness of the nano-conjugated construct on enhancement of reproductive output. In a study Rather *et al.* (2013) found the changes in gonadal maturity after delivery of nano-conjugated Luteinizing Hormone-Releasing Hormone (LHRH) hormone in *Cyprinus carpio.* The toxicity of chitosan nanoparticles was analyzed in liver tissues to rule out any possible toxicity of the chitosan nanoparticle. The histological examination of the liver tissue did not show any cellular disruption and haemorrhage. The hepatocytes were found to be intact and nucleus was clearly visible. There were no visible changes in the histological architecture of the liver tissue in different fish injected with chitosan nano-conjugated plasmid and naked plasmid. The present study indicates that chitosan did not create any toxicity to *C. batrachus* at 0.2mg/ml. Chitosan, a natural polymer is obtained by alkaline deacetylation of chitin and it is nontoxic, biocompatible, and biodegradable (Jayakumar, *et al*., 2010). Many researchers have found very low cytotoxicity of chitosan nanoparticles by MTT assay in different fish cell lines (Rajeshkumar *et al.*, 2009; Vimal *et al.*, 2012; Vimal *et al.*, 2013).

6. SUMMARY

In India catfishes are the second most commercially important cultured species followed by freshwater carps. Amongst the catfishes *Clarias batrachus* is an economically important species, due to its good taste and medicinal value. But seed availability of *C. batrachus* is a major concern for aquaculture production due to hurdles in captive breeding. Currently GnRH and pituitary extract are used for induced breeding in which female are stripped after injection but male always needs to be sacrificed for obtaining the milt and this is not desirable as we end up in losing the animal. To find remedy to this problem many researchers across the world have focussed on studying the morphological features like deep location of testis in body cavity, milt accumulation on the convex lobular edge of testis and seminal vesicle surrounding by thick interstitial tissue etc. But the molecular basis of these reasons are not clear. There is strong possibility to resolve this problem by understanding molecular expression pattern of different reproductive genes and their products that control the sexual development and reproduction in fishes. Steroid hormones are the key factors in sexual maturation and reproduction. The production of these hormones depends on steroidogenic acute regulatory protein (StAR) gene expression. StAR protein transports cholesterol, a precursor for all steroid hormones. StAR is a key gene in steroid hormone production pathway and is proposed to play an important role sexual development *C. batrachus.* To understand its functional role we developed chitosan conjugated StAR gene construct of *Clarias batrachus* and determined the effects of chitosan conjugated StAR gene constructs on hormonal profile of *Clarias batrachus* and also studied the expression level of different reproductive genes after nanodelivery of chitosan conjugated StAR gene construct by qRT-PCR.

Mature male *C. batrachus* specimen were collected from CIFA, Bhubaneswar and testes were dissected out. Total RNA was isolated and the StAR gene was amplified and cloned in pcDNA4/HisMax A vector. The construct pcDNA4-StARorf was transformed in *E.coli* DH5α strain and confirmed by sequencing. To confirm the expression of construct it was transfected in striped snake head (SSN-1) cell lines and analysed by SDS-PAGE and western blotting. In SDS-PAGE a 30 kDa band was clearly observed and gave positive results on western blotting. For *in vivo* study the plasmid was conjugated with chitosan nanoparticles of mean size 135.4 nm and zeta potential 17.7 mV. After the conjugation the stability was checked by gel retardation assay. The stability of the construct was confirmed after it remained firm on digestion with DNase.

The construct delivery experiment was conducted at CIFE, Kakinada centre. Two treatment groups TR1, TR2 along with 2 control groups CTL1 and CTL2 were designed. Two cement pond per treatment and control group were maintained with 10 males per pond. Animals in group TR1 were injected intramuscularly with chitosan conjugated pcDNA4-StARorf, in TR2 with naked pcDNA4-StARorf, in CTL1 with blank chitosan nanoparticles and in CTL2 with empty vector. Five time points were taken viz. 12h, 24h, 48h, 72h and 120h and at each time point 2-2 animals were collected from all the ponds. Tissue distribution analysis by CMV based primers shows the transportation of construct to different tissues like testis, head kidney, brain and muscle. The expression of five reproductive gene Cyp11a1, Cyp17a1, 3β-HSD, 17β-HSD and Cytochrome P450 aromatase were studied with real time PCR. Cyp11a1 expression was increased highest 10 folds and 17β-HSD lowest 4.2 fold compare to control at 5th day and aromatase gene expression was also increased to 4.7 fold. All 5 genes showed maximum expression at 5th day. This expression pattern of downstream genes indicates the clear role of StAR gene in steroid hormones production.

In the present study, the level of 11-ketotestosterone showed increasing trend after 72 hours of injection. Whereas the level of estradiol increased initially but there was no significant difference ($p<0.05$) found at 5th day. The increase in hormonal level shows the efficacy of construct but the changes are not much accountable. In TR1 group the histological analysis of testis tissue showed the increase in primary spermatogonial cells on the wall of seminiferous tubules and the number of spermatozoa also increased in the seminiferous tubules at 10th day whereas in TR2 group there was no significance difference. From the liver tissue sectioning it can be concluded there was no cytotoxicity of chitosan nanoparticles at 0.2mg/ml in *C. batrachus.*

The following conclusions may be drawn from the present study-

- StAR gene construct from *C. batrachus* was developed for the first time.
- Chitosan nanoparticles conjugated StAR gene construct showed stability against the nucleases while the naked construct was digested.
- In vitro transfection confirm the functionality of construct in biological systems.
- Expression level of selected reproductive genes increase significantly under the influence of nano-conjugated construct.
- Hormonal and histology analysis shows the role of StAR gene in fish reproduction.
- No toxicity was observed due to chitosan nanoparticles in the present study.

The expression pattern of most of the genes was in increasing trend with respect to time so the further study may be conducted to check maximum expression. Further plasmid persistence study may be conducted. Direct delivery of nano-conjugated StAR protein may be useful in solving the reproductive dysfunction in the fish.

7. REFERENCES

Aceves-Ramos, A., de la Torre, P., Hinojosa, L., Ponce, A., García-Villegas, R., Laclette, J. P. and Romano, M. C., 2014. Cloning, characterization and functional expression of *Taenia solium* 17 beta-hydroxysteroid dehydrogenase. *General and comparative endocrinology*, 203:186-192.

Aesoy, R., Mellgren, G., Morohashi, K. I. and Lund, J., 2002. Activation of cAMP-dependent protein kinase increases the protein level of steroidogenic factor-1. *Endocrinology*, 143(1):295-303.

Aparicio, S., Chapman, J., Stupka, E., Putnam, N., Chia, J. M., Dehal, P. and Brenner, S., 2002. Whole-genome shotgun assembly and analysis of the genome of *Fugu rubripes*. *Science*, 297(5585):1301-1310.

Arakane, F., Kallen, C. B., Watari, H., Foster, J. A., Sepuri, N. B. V., Pain, D. and Strauss, J. F. 1998. The mechanism of action of steroidogenic acute regulatory protein (StAR) StAR acts on the outside of mitochondria to stimulate steroidogenesis. *Journal of Biological Chemistry*, 273(26): 16339-16345.

Arakane, F., Sugawara, T., Nishino, H., Liu, Z., Holt, J. A., Pain, D. and Strauss, J. F., 1996. Steroidogenic acute regulatory protein (StAR) retains activity in the absence of its mitochondrial import sequence: implications for the mechanism of StAR action. *Proceedings of the National Academy of Sciences*, 93(24):13731-13736.

Aspden, T., Illum, L. and Skaugrud, O., 1996. Chitosan as a nasal delivery system: evaluation of insulin absorption enhancement and effect upon nasal membrane integrity using rat a models. *European Journal of Pharmaceutical Sciences* 4: 23–31.

Bain, P. A., Yoo, M., Clarke, T., Hammond, S. H. and Payne, A. H., 1991. Multiple forms of mouse 3 beta-hydroxysteroid dehydrogenase/delta 5-delta 4 isomerase and differential expression in gonads, adrenal glands, liver, and kidneys of both sexes. *Proceedings of the National Academy of Sciences*, 88(20):8870-8874.

Bauer, M. P., Bridgham, J. T., Langenau, D. M., Johnson, A. L. and Goetz, F. W., 2000. Conservation of steroidogenic acute regulatory (StAR) protein structure and expression in vertebrates. *Molecular and cellular endocrinology*, 168(1), 119-125.

Blasco, M., Fernandino, J.I., Guilgur, L.G., Strussmann, C.A., Somoza, G.M.and Vizziano-Cantonnet, D., 2010. Molecular characterization of cyp11a1 and cyp11b1 and their gene expression profile in pejerrey (Odontesthes bonariensis) during early gonadal development. *Comp. Biochem. Physiol. A.,* 156:110–118.

Borg, B., 1994. Androgens in teleost fishes. *Comp Biochem Physiol C Pharmacol Toxicol Endocrinol.,* 109: 219–45

Boudreau, R. L. and Davidson, B. L., 2012. Generation of hairpin-based RNAi vectors for biological and therapeutic application. *Methods Enzymol.,* 507: 275-296.

Bozkir, A. and Saka, O.M., 2004a. Chitosan–DNA nanoparticles: effect on DNA integrity, bacterial transformation and transfection efficiency. *Journal of Drug Targeting* 12:281–288.

Brion, F., Le Page, Y., Piccini, B., Tong, S. K., Chung, B. C., and Kah, O. (2012). Brain aromatase (Cyp19a1b) is a highly sensitive gene to estrogens and xeno-estrogens. In Endocrine Disruptors 2012 (p. NC). Smithers. Shropshire.

Callard, I.P. *et al*., 1978 Testicular regulation in non-mammalian vertebrates. *Biol. Reprod.,* 18:16-43

Capan, Y., Woo, B.H., Gebrekıdan, S., Ahmed, S. and Deluca, P., 1999. Preparation and characterization of poly (D, L-lactide-co-glycolide) microspheres for controlled release of poly (L-lysine) complexed plasmid DNA. *Pharmaceutical Research* 16: 509–513.

Caron, K. M., Soo, S. C., Wetsel, W. C., Stocco, D. M., Clark, B. J. and Parker, K. L., 1997. Targeted disruption of the mouse gene encoding steroidogenic acute regulatory protein provides insights into congenital lipoid adrenal hyperplasia. *Proceedings of the National Academy of Sciences*, 94(21):11540-11545.

Cavaco, J.E.B., Vilrokx, C.C., Trudeau, V.L., Schulz, R.W. and Goos, H.J.T.,1998. Sex steroids and the initiation of puberty in male African catfish (*Clarias gariepinus*). *Am J Physiol Regul Integr Comp Physiol*., 275:1793 – 802.

Chung, B., Guo, I.C. and Chou, S.J., 1997. Transcriptional regulation of the CYP11A1 and ferredoxin genes. *Steroids*, 62: 37–42.

Corbeil, S., Kurath, G. and Lapatra, S. E., 2000. Fish DNA vaccine against infectious hematopoietic necrosis virus: efficacy of various routes of immunization. *Fish and shellfish immunology*, 10(8):711-723.

Corsi, K., Chellat, F., Yahia, L. and Fernandes. J.C., 2003. Mesenchymal stem cells, MG63 and HEK293 transfection using chitosan–DNA nanoparticles, *Biomaterials*, 24:1255–1264.

Courtenay, J.W.R. and Hensley, D.A., 1980. Special problems associated with monitoring exotic species. *Biological monitoring of fish*, pp: 281-307.

Courtenay, W.R., Jr., Sahlman, H.F., Miley, W.W. and D.J. Herrema. 1974. Exotic fishes in fresh and brackish waters of Florida. *Biological Conservation,* 6:292-302.

Crim, L. W., Peter, R. E. and Billard, R., 1976. Stimulation of gonadotropin secretion by intraventricular injection of hypothalamic extracts in the goldfish, *Carassius auratus*. *General and comparative endocrinology*, 30(1):77-82.

Cui, Z. and Mumper. R.J., 2001 Chitosan-based nanoparticles for topical genetic immunization, *J. Control.* 75:409–419.

Das, S. K. 2002. Seed production of Magur (*Clarias batrachus*) using a rural model portable hatchery in Assam, India-A farmer proven technology. *Aquaculture Asia*, 7(2):19-21.

De Leeuw R., Goods, H.J.T., Richter, C.J.J. and Edind, E.H., 1985. Pimozide-LHRHa induced breeding in the African catfish, *Clarias gariepinus* (Burchell). *Aquaculture, 44: 299-302*

Debnath, S., 2011. *Clarias batrachus*, the medicinal fish: An excellent candidate for aquaculture and employment generation. *In International Conference on Asia Agriculture and Animal*. IPCBEE (13).

Diotel, N., Do Rego, J. L., Anglade, I., Vaillant, C., Pellegrini, E., Gueguen, M. M.and Kah, O., 2011. Activity and expression of steroidogenic enzymes in the brain of adult zebrafish. *European Journal of Neuroscience,* 34(1): 45-56.

Durand, S., Lightner, D. V., Redman, R. M. and Bonami, J.R., 1997. Ultrastructure and morphogenesis of white spot syndrome baculovirus (WSSV).*Diseases of Aquatic Organisms*, 29(3): 205-211.

Evans, D.H. (Ed.) 1998. The physiology of fishes. Second edition. CRC Marine Science Series, 9. CRC Press: Boca Raton. ISBN 0-8493-8427-3. 519 pp.

Garnett, M.C., 1999. Gene delivery systems using cationic polymers. *Critical Reviews in Therapeutic Drug Carrier* Systems 16:147–207.

Goetz, F. W., Norberg, B., McCauley, L. A. and Iliev, D. B., 2004. Characterization of the cod (*Gadus morhua*) steroidogenic acute regulatory protein (StAR) sheds light on StAR gene structure in fish. *Comparative Biochemistry and Physiology* Part B: Biochemistry and Molecular Biology,137(3), 351-362.

Goldstone, J.V., McArthur, A.G., Kubota, A., Zanette, J., Parente, T., Jonsson, M.E., Nelson, D.R. and Stegeman, J.J., 2010. Identification and developmental expression of the full complement of cytochrome P450 genes in zebrafish. *BMC Genomics* 11: 643.

Gomez, C.M., Livingston, S. K., Muro-Cacho, C., Sanders, S. and Levine, R. P., 1996. Introduction of foreign genes into the tissue of live fish by direct injection and particle bombardment. *Dis. Aquat. Org.,* 27: 5-12

Gonçalves, D. and Oliveira, R. F., 2010. Hormones and sexual behavior of teleost fishes. *Hormones and reproduction of vertebrates*, 7: 119-147.

Grenha, A., 2012. Chitosan nanoparticles: a survey of preparation methods. *Jour. of drug targt*. 20(4):291-300.

Guiguen, Y., Chiang, E., Yan, Y.L., Postlethwait, J. and Chung, B.C., 2001. Two Cyp19 (P450 aromatase) genes on duplicated Zebra fish chromosomes are expressed in ovary or brain. *Molecular biology and evolution*, 18(4): 542-550.

Guraya, S. S., 1976. Recent advances in the morphology, histochemistry and biochemistry of steroid-synthesizing cellular sites in the testes of nonmammalian vertebrates. *Int. Rev. Cytol*., 47: 99-136.

Hagen, I. J., Kusakabe, M. and Young, G., 2006. Effects of ACTH and cAMP on steroidogenic acute regulatory protein and P450 11β-hydroxylase messenger RNAs in rainbow trout interrenal cells: relationship with in vitro cortisol production. *General and comparative endocrinology*, 145(3):254-262.

Hansen, E., Fernandes, K., Goldspink, G., Butterworth, P., Umeda, P. K.and Chang, K. C., 1991. Strong expression of foreign genes following direct injection into fish muscle. *FEBS letters*, 290(1):73-76.

Hatano, O., Takayama, K., Imai, T., Waterman, M.R., Takakusu, A., Omura, T. and Morohashi, K., 1994. Sex-dependent expression of a transcription factor, Ad4BP, regulating steroidogenic P-450 genes in the gonads during prenatal and postnatal rat development. *Development,* 120: 2787–2797.

Hinfray, N., Baudiffier, D., Leal, M. C., Porcher, J. M., Ait-Aissa, S., Le Gac, F. and Brion, F., 2011.Characterization of testicular expression of P450 17α-hydroxylase, 17, 20-lyase in zebrafish and its perturbation by the pharmaceutical fungicide clotrimazole. *General and comparative endocrinology*, 174(3): 309-317.

Hirosue, S., Muller, B.G., Mulligan, R.C., Langer, R., 2001. Plasmid DNA encapsulation and release from solvent diffusion nanospheres. Journal of Controlled Release 70:231–242.

Hossain, Q., Hossain, M. A., and Parween, S., 2006. Artificial breeding and nursery practices of *Clarias batrachus* (Linnaeus, 1758). *Sci World*, 4(4), b10.

Idler, D. R., Schmidt, P. J. and Ronald, A. P., 1960. Isolation and identification of 11-ketotestosterone in salmon plasma. *Canadian journal of biochemistry and physiology.* 38(10): 1053-1057.

Ikeda, Y., Shen, W.H., Ingraham, H.A. and Parker, K.L., 1994. Developmental expression of mouse steroidogenic factor-1, an essential regulator of the steroid hydroxylases. *Mol. Endocrinol*., 8: 654–662.

Illum, L., Jabbal-Gill, I., Hinchcliffe, M., Fisher, A.N., Davis, S.S., 2001 Chitosan as a novel nasal delivery system for vaccines, *Adv. Drug Deliv. Rev.* 51: 81–96.

Jaillon, O., Aury, J. M., Brunet, F., Petit, J. L., Stange-Thomann, N., Mauceli, E. and Nusbaum, C., 2004. Genome duplication in the teleost fish *Tetraodon nigroviridis* reveals the early vertebrate proto-karyotype. *Nature*, 431(7011): 946-957.

Jayakumar, R., Prabaharan, M., Nair, S. V., Tokura, S., Tamura, H. and Selvamurugan, N., 2010. Novel carboxymethyl derivatives of chitin and chitosan materials and their biomedical applications. *Progress in Materials Science*, 55(7):675-709.

Jeng, S. R., Pasquier, J., Yueh, W. S., Chen, G. R., Lee, Y. H., Dufour, S. and Chang, C. F., 2012. Differential regulation of the expression of cytochrome P450 aromatase, estrogen and androgen receptor subtypes in the brain–pituitary–ovarian axis of the Japanese eel (*Anguilla japonica*) reveals steroid dependent and independent mechanisms. *General and comparative endocrinology*, 175(1), 163-172.

K.A. Janes, P. Calvo and M.J. Alonso., 2001. Polysaccharide colloidal particles as delivery systems for macromolecules, *Adv. Drug Deliv. Rev.* 47:83–97.

Kallen, C. B., Billheimer, J. T., Summers, S. A., Stayrook, S. E., Lewis, M. and Strauss, J. F., 1998. Steroidogenic acute regulatory protein (StAR) is a sterol transfer protein. *Journal of Biological Chemistry*, 273(41): 26285-26288.

Kasahara, M., Naruse, K., Sasaki, S., Nakatani, Y., Qu, W., Ahsan, B. and Kohara, Y., 2007. The medaka draft genome and insights into vertebrate genome evolution. *Nature*, 447(7145): 714-719.

Kazeto, Y., Ijiri, S., Todo, T., Adachi, S. and Yamauchi, K., 2000. Molecular cloning and characterization of Japanese eel ovarian P450c17 (CYP17) cDNA. *General Comparative Endocrinology*, 118: 123-133.

Kime, D. E., 1993. ‘Classical’ and ‘non-classical’ reproductive steroids in fish. *Reviews in Fish Biology and Fisheries*, 3(2):160-180.

Kitano, T., Takamune, K., Nagahama, Y. and Abe, S. I., 2000. Aromatase inhibitor and 17a-methyltestosterone cause sex-reversal from genetical females to phenotypic males and suppression of P450 aromatase gene expression in Japanese flounder (*Paralichthys olivaceus*). *Mol Reprod Dev.,* 56(1): 1-5.

Kobayashi, T., Chang, X. T., Nakamura, M., Kajiura, H. and Nagahama, Y., 1996. Fish 3β-Hydroxysteroid Dehydrogenase/Δ5-Δ4Isomerase: Antibody Production and Their Use for the Immunohistochemical Detection of Fish Steroidogenic Tissues. *Zoological science*, 13(6): 909-914.

Kumar, R. S., Ijiri, S and Trant, J. M., 2000. Changes in the expression of genes encoding steroidogenic enzymes in the channel catfish (*Ictalurus punctatus*) ovary throughout a reproductive cycle. *Biology of Reproduction*, 63(6):1676-1682.

Kusakabe, M., Todo, T., McQuillan, H. J., Goetz, F. W. and Young, G., 2002. Characterization and expression of steroidogenic acute regulatory protein and MLN64 cDNAs in trout. *Endocrinology*, 143(6), 2062-2070.

Lavertu, M., Methot, S., Tran Khanh, N. and Buschmann, M.D., 2006. High efficiency gene transfer using chitosan/DNA nanoparticles with specific combinations of molecular weight and degree of deacetylation. *Biomaterials* 27: 4815–4824.

Leong, K.W., Mao, H.Q., Trung Le, V.L., Roy, K., Walsh, S.M., August, J.T., 1998. DNA-polycationnanospheres as non-viral gene delivery vehicles. *Journal of Controlled Release* 53:183–193.

Li, Y. Y., Inoue, K. and Takei, Y., 2003. Steroidogenic acute regulatory protein in eels: cDNA cloning and effects of ACTH and seawater transfer on its mRNA expression. *Zoological science*, 20(2), 211-219.

Lin, D., Sugawara, T., Strauss, J., Clark, B. J., Stocco, D. M., Saenger, P. and Miller, W. L., 1995. Role of steroidogenic acute regulatory protein in adrenal and gonadal steroidogenesis. *Science*, 267(5205): 1828-1831.

Lorence, M. C., Murry, B. A., Trant, J. M. and Mason, J. I., 1990. Human 3β-Hydroxysteroid Dehydrogenase/δ5→ 4Isomerase from Placenta: Expression in Nonsteroidogenic Cells of a Protein that Catalyzes the Dehydrogenation/Isomerization of C21 and C19 Steroids*. *Endocrinology*.126 (5): 2493-2498.

Lorence, M. C., Naville, D., Graham-Lorence, S. E., Mack, S. O., Murry, B. A., Trant, J. M. and Mason, J. I., 1991. 3β-Hydroxysteroid dehydrogenase/Δ 5→ 4-isomerase expression in rat and characterization of the testis isoform. *Molecular and cellular endocrinology*, 80(1): 21-31.

Lukacik, P., Kavanagh, K. L. and Oppermann, U. 2006. Structure and function of human 17β-hydroxysteroid dehydrogenases. *Molecular and cellular endocrinology*, 248(1): 61-71.

Mahapatra, B. K., 2004. Conservation of the Asiatic catfish, *Clarias batrachus* through artificial propagation and larval rearing technique in India. *Aquaculture Asia*, *9*(4), 8-9.

Manickam, P. and Joy, K. P., 1989. Induction of maturation and ovulation by pimozide –LHRH analogue treatment and resulting high quality egg production in the Asian catfish, *Clarias batrachus* (Linn.). *Aquaculture*, 83:193-199.

Manna, P. R. and Stocco, D. M., 2005. Regulation of the steroidogenic acute regulatory protein expression: functional and physiological consequences. Current Drug Targets-Immune, *Endocrine and Metabolic Disorders*, 5(1):93-108.

Manna, P. R., Eubank, D. W., Lalli, E., Sassone C. P. and Stocco, D. M., 2003. Transcriptional regulation of the mouse steroidogenic acute regulatory protein gene by the cAMP response-element binding protein and steroidogenic factor 1. *Journal of molecular endocrinology*, 30(3): 381-397.

Manna, Pulak R., et al., 2006 "cAMP-independent signaling regulates steroidogenesis in mouse Leydig cells in the absence of StAR phosphorylation." *Journal of molecular endocrinology,* 37(1): 81-95.

Masterson, J., 2007. *Clarias batrachus*. Smithsonian Marine Station of Fort Pierce. http://www.sms.si.edu/irlspec/clarias_batrachus.htm.

Matsumine, H., Hirato, K., Yanaihara, T., Tamada, T. and Yoshida, M., 1986. Aromatization by skeletal muscle. *The Journal of Clinical Endocrinology and Metabolism,* 63(3): 717-720.

Mazumdar, M., Sakharkar, A.J., Singru, P. and Subheda, N., 2007. Reproduction phase-related variations in neuropeptide Y immunoreactivity in the olfactory system, forebrain, and pituitary of the female catfish, *Clarias batrachus* (Linn.). *Journal of Comparative Neurology, 504(4):* 450-469.

Meyer, T. E. and Habener, J. F., 1993. Cyclic Adenosine 3′, 5′-Monophosphate Response Element Binding Protein (CREB) and Related Transcription-Activating Deoxyribonucleic Acid-Binding Proteins*. *Endocrine reviews*, 14(3):269-290.

Mindnich, R., Deluca, D. and Adamski, J., 2004.Identification and characterization of 17β-hydroxysteroid dehydrogenases in the zebrafish, *Danio rerio*. *Molecular and cellular endocrinology*, 215(1): 19-30.

Miura, C., Miura,T., Yamashita, M., Yamauchi K .and Nagahama Y., 1996.Hormonal induction of all stages of spermatogenesis in germ-somatic cell coculture from immature Japanese eel testis. *Dev Growth Differ*. 38:257–62.

Morris, D. J., Brem, A. S., Ge, R., Jellinck, P. H., Sakai, R. R. and Hardy, M. P., 2003. The functional roles of 11β-HSD1: vascular tissue, testis and brain. *Molecular and cellular endocrinology*, 203(1): 1-12.

Muir, J., 2005. Managing to harvest, Prospect on the potential of aquaculture, *Phil. Trans.R. Soc*., 360: 191-218.

Mumper, R.J., Wang, J.J., Claspell, J.M. and Rolland, A.P., 1995. Novel polymeric condensing carriers for gene delivery. *Proceedings of the International Symposium on Controlled Release Bioactive Materials* 22:178–179.

Nematollahi, M. A., VanPelt, H., Atsma, W.K, J., 2012. High levels of corticosterone, and gene expression of star, cyp17a2, hsd3b, cyp21, hsd11b2 during acute stress in common carp with interrenal hyperplasia. *General and comparative endocrinology*, 176(2):252-258.

Nobel, S., Abrahmsen, L., and Oppermann, U., 2001. Metabolic conversion as a pre‐receptor control mechanism for lipophilic hormones. *European Journal of Biochemistry*, 268(15): 4113-4125.

Nunez, B. S. and Evans, A. N., 2007. Hormonal regulation of the steroidogenic acute regulatory protein (StAR) in gonadal tissues of the Atlantic croaker (*Micropogonias undulatus*). *General and comparative endocrinology*, 150(3): 495-504.

Pan, Y., Li, Y. and Zhao, H., 2002. Bioadhesive polysaccharide in protein delivery system: chitosan nanoparticles improve the intestinal absorption of insulin in vivo. *International Journal of Pharmaceutics* 249:139–147.

Panyam, J. and Labhasetwar, V., 2003 Biodegradable nanoparticles for drug and gene delivery to cells and tissue, *Adv. Drug Deliv. Rev.,* 55:329–347.

Parajes, S., Griffin, A., Taylor, A.E., Rose, I.T., Miguel-Escalada, I., Hadzhiev, Y., Arlt, W., Shackleton, C., Müller, F. and Krone, N., 2013. Redefining the initiation and maintenance of zebrafish interrenal steroidogenesis by characterizing the key enzyme Cyp11a2. *Endocrinology*, 154:2702–2711.

Pathan, M., Gireesh-Babu, P., Pavan-Kumar, A., Jeena, K., Sharma, R., Makesh, M. and Krishna, G., 2013. In vivo therapeutic efficacy of recombinant *Penaeus monodon* antiviral protein (rPmAV) administered in three different forms to WSSV infected *Penaeus monodon*. *Aquaculture*, 376, 64-67.

Payne, A. H. and Hales, D. B., 2004. Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones. *Endocrine reviews,* 25(6): 947-970.

Ponting, C. P. and Aravind, L., 1999. START: a lipid-binding domain in StAR, HD-ZIP and signalling proteins. *Trends in biochemical sciences*, 24(4):130-132.

Rajakumar, A. and Senthilkumaran, B., 2014. Expression analysis of cyp11a1 during gonadal development, recrudescence and after hCG induction and sex steroid analog treatment in the catfish, *Clarias batrachus*. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 176: 42-47.

Rajesh Kumar, S., Ishaq Ahmed, V.P., Parameswaran, V., Sudhakaran, R., Sarath Babu, V., Sahul Hameed, A.S., 2008. Potential use of chitosan nanoparticles for oral delivery of DNA vaccine in Asian sea bass (*Lates calcarifer*) to protect from *Vibrio anguillarum* (Listonella*). Fish and Shellfish Immunology* 25: 47–56.

Rajeshkumar, S., Venkatesan, C., Sarathi, M., Sarathbabu, V., Thomas, J., Basha, K. A. and Hameed, A. S., 2009. Oral delivery of DNA construct using chitosan nanoparticles to protect the shrimp from white spot syndrome virus (WSSV). *Fish and shellfish immunology*, 26(3), 429-437.

Ramya, V. L., Sharma, R., Gireesh‐Babu, P., Patchala, S. R., Rather, A., Nandanpawar, P. C. and Eswaran, S., 2014. Development of chitosan conjugated DNA vaccine against nodavirus in *Macrobrachium rosenbergii* (De Man, 1879). *Journal of fish diseases*, 37(9):815-824.

Rao G.R.M., Singh S.K. and A.K. Sahu., 1995. Fry and fingerling production of *Clarias batrachus* (Linnaeus). *Asian J. Zool. Sci*., 4:7-10.

Rather, M. A., Sharma, R., Gupta, S., Ferosekhan, S., Ramya, V. L. and Jadhao, S. B., 2013. Chitosan-nanoconjugated hormone nanoparticles for sustained surge of gonadotropins and enhanced reproductive output in female fish. PloS one, 8(2), e57094.

Richards, J.S., Jahnsen, T., Hedin, L., Lifka,J., Ratoosh, S., Durica,J.M., and Goldring, N.B.,1987. Ovarian follicular development: From physiology to molecular biology. *Recent Prog Horm Res* 43:231-276.

Richardson, S.C.W., Kolbe, H.V.J. and Duncan. R., 1999. Potential of low molecular mass chitosan as a DNA delivery system: biocompatibility, body distribution and ability to complex and protect DNA, *Int. J. Pharm.* 178: 231–243.

Roderick, S. L., Chan, W. W., Agate, D. S., Olsen, L. R., Vetting, M. W., Rajashankar, K. R. and Cohen, D. E., 2002. Structure of human phosphatidylcholine transfer protein in complex with its ligand. *Nature Structural and Molecular Biology*, 9(7):507-511.

Romoren, K., Thu, B. J. and Evensen, O., 2004. Expression of luciferase in selected organs following delivery of naked and formulated DNA to rainbow trout (*Oncorhynchus mykiss*) by different routes of administration. *Fish and shellfish immunology*, 16(2): 251-264.

Romoren, K., Thu, B.J. and Evensen, O., 2002. Immersion delivery of plasmid DNA. II. A study of the potentials of a chitosan based delivery system in rainbow trout (*Oncorhynchus mykiss*) fry. *Journal of Controlled Release* 85:215–225.

Ruane, N. M., Lambert, J. G. D., Goos, H. T. and Komen, J., 2005. Hypocorticism and interrenal hyperplasia are not directly related to masculinization in XX mas− 1/mas− 1 carp, *Cyprinus carpio. General and comparative endocrinology,* 143(1): 66-74.

Sakai, N., Tanaka, M., Adachi, S., Miller, W.L. and Nagahama, Y., 1992. Rainbow trout cytochrome P-450c17 (17α-hydroxylase/17, 20-lyase)-cDNA cloning, enzymatic properties and temporal pattern of ovarian P-450c17 mRNA expression during oogenesis. *FEBS Letter*, 301:60-64.

Sambrook, S.J., Russel, D.W., Janssen K.A. and Irwuin, N.J., 2001. *Molecular Cloning, A Laboratory Manual (*Third Editions), Cold Spring Harbor Laboratory Press

Saranya, N., Moorthi, A., Saravanan, S., Devi, M. P. and Selvamurugan, N. 2011. Chitosan and its derivatives for gene delivery. *International journal of biological macromolecules*, *48*(2):234-238.

Sassone-Corsi, P., 1995. Transcription factors responsive to cAMP. *Annual review of cell and developmental biology*, 11(1):355-377.

Schmittgen, T. D. and Livak, K. J., 2008. Analyzing real-time PCR data by the comparative CT method. *Nature protocols*, 3(6):1101-1108.

Schreibman M.P., Margolis N.H., Halpern-Sebold L.R., Goos H.J.T., Perlman, P.W., 1986. The influence of androgen administration on the structure and function of the brain–pituitary–gonad axis of sexually immature platyfish, *Xiphophorus maculatus*. *Cell Tissue Res.,* 245:519–24.

Schultz, D. R., Perez, N., Tan, C. K., Mendez, A. J., Capo, T. R., Snodgrass, D. and Serafy, J. E., 2005. Concurrent levels of 11‐ketotestosterone in fish surface mucus, muscle tissue and blood. *Journal of Applied Ichthyology*, 21(5): 394-398.

Sharma, R., Rather, M. A., Vijaykumar Leela, R., Saha, H., Purayil, P., Babu, S., and Munilkumar, S., 2014. Preliminary observations on effect of nano‐conjugated pheromones on *Clarias batrachus* (Linnaeus, 1758). *Aquaculture Research*, 45(8):1415-1420.

Simon, R.H., Engelhardt, J.F., Yang, Y., Zepeda, M., Weber P.S., Grossman, M. and Wilson, J.M., 1993. Adenovirus-mediated gene transfer of the CFTR gene to the lung of non-human primates: a toxicity study. *Human Gene* Therapy 4:771–780.

Sivik, T., Vikingsson, S., Gréen, H. and Jansson, A., 2012. Expression Patterns of 17β-Hydroxysteroid Dehydrogenase 14 in Human Tissues. Hormone and Metabolic Research, 44(13): 949-956.

Soccio, R. E. and Breslow, J. L., 2003. StAR-related lipid transfer (START) proteins: mediators of intracellular lipid metabolism. *Journal of Biological Chemistry*, 278(25): 22183-22186.

Sreenivasulu, G., and Senthilkumaran, B., 2009a. A role for cytochrome P450 17α-hydroxylase/c17-20 lyase during shift in steroidogenesis occurring in ovarian follicles prior to oocyte maturation. *The Journal of steroid biochemistry and molecular biology*, 115(3): 77-85.

Sreenivasulu, G., Sridevi, P., Sahoo, P. K., Swapna, I., Ge, W., Kirubagaran, R. and Senthilkumaran, B., (2009b). Cloning and expression of StAR during gonadal cycle and hCG-induced oocyte maturation of air-breathing catfish, *Clarias gariepinus.* Comparative Biochemistry and Physiology Part B: *Biochemistry and Molecular Biology*, 154(1): 6-11.

Sridevi, P., Chaitanya, R. K., Prathibha, Y., Balakrishna, S. L., DuttaGupta, A., and Senthilkumaran, B., 2015. Early exposure of 17α‐ethynylestradiol and diethylstilbestrol induces morphological changes and alters ovarian steroidogenic pathway enzyme gene expression in catfish, *Clarias gariepinus*. *Environ Toxicol.* (4):439-51.

Stocco, D. M. and Clark, B. J., 1996. Regulation of the Acute Production of Steroids in Steroidogenic Cells*. *Endocrine reviews*, 17(3): 221-244.

Stocco, D. M., 2000. The role of the StAR protein in steroidogenesis: challenges for the future." *Journal of Endocrinology* 164(3): 247-253.

Sugawara, T., Kiriakidou, M., McAllister, J. M., Holt, J. A., Arakane, F. and Strauss, J. F., 1997. Regulation of expression of the steroidogenic acute regulatory protein (StAR) gene: a central role for steroidogenic factor 1.*Steroids*, 62(1):5-9.

Sugawara, T., Lin, D., Holt, J.A., Martin, K. O., Javitt, N. B., Miller, W. L. and Strauss III, J. F., 1995. Structure of the human steroidogenic acute regulatory (StAR) protein gene: StAR stimulates mitochondrial cholesterol 27-hydroxylase. *Biochemistry,* 34(39), 12506-12512.

Talwar, P. K., 1991. Inland fishes of India and adjacent countries (Vol. 2). CRC Press.

Tamaoki, B., 1980. Steroids and their Mechanism of Action in Nonmammalian Vertebrates (pp. 1-15). Raven Press New York.

Tan-Fermin J. D., Pagador, R.R. and Chavez,R.C., 1997. LHRHa and pimozide-induced spawning of Asian catfish *Clarias macrocephalus* (Gunther) at different times during an annual reproductive cycle. *Aquaculture*, 148:323-31.

Teng, H., Cai, W., Zeng, K., Mao, F., You, M., Wang, T. and Sun, Z., 2013. Genome-wide identification and divergent transcriptional expression of StAR-related lipid transfer (START) genes in teleosts. *Gene,* 519(1): 18-25.

Teugels, G. G., 1996. Taxonomy, phylogeny and biogeography of catfishes (Ostariophysi, Siluroidei): an overview. *Aquatic Living Resources*, 9(S1):9-34.

Tsujishita, Y. and Hurley, J. H., 2000. Structure and lipid transport mechanism of a StAR-related domain. *Nature Structural and Molecular Biology*, 7(5): 408-414.

Van den Hurk, R., Vermeij, J.A.J., Stegenga, J., Peute, J. and Van Oordt, P.G.W.J., 1978. Cyclic changes in the testis and vas deferens of the rainbow trout (*Salmo gairdneri*) with special reference to sites of steroidogenesis. *InAnnales de Biologie Animale Biochimie Bio physique*. 18 (4):899-904

Van Dyk, J. C. and Pieterse, G. M., 2008. A histomorphological study of the testis of the sharptooth catfish (*Clarias gariepinus*) as reference for future toxicological assessments. *Journal of Applied Ichthyology*, 24(4): 415-422.

Vimal, S., Majeed, S. A., Taju, G., Nambi, K. S. N., Raj, N. S., Madan, N. and Hameed, A. S., 2013. Chitosan tripolyphosphate (CS/TPP) nanoparticles: preparation, characterization and application for gene delivery in shrimp. *Acta tropica*, 128(3), 486-493.

Vimal, S., Taju, G., Nambi, K. S. N., Majeed, S. A., Babu, V. S., Ravi, M. and Hameed, A. S., 2012. Synthesis and characterization of CS/TPP nanoparticles for oral delivery of gene in fish. *Aquaculture*, 358, 14-22.

Vinagradov, S.V., Bronich, T.K. and Kabanov, A.V., 2002. Nanosized cationic hydrogels for drug delivery: preparation, properties and interactions with cells, *Adv. Drug Deliv. Rev*. 54:223–233.

Wang, S.L., Yao, H.H., Guo, L.L., Dong, L., Li, S.G., Gu, Y.P. and Qin, Z.H., 2009. Selection of optimal sites for TGFB1 gene silencing by chitosan–TPP nanoparticle-mediated delivery of shRNA. *Cancer Genetics and Cytogenetics* 190:8–14.

Wang, Y., and Ge, W., 2004. Cloning of zebrafish ovarian P450c17 (CYP17, 17α-hydroxylase/17, 20-lyase) and characterization of its expression in gonadal and extra-gonadal tissues. General and comparative endocrinology, 135(2): 241-249.

Waterman, M. R., 1994. Biochemical diversity of cAMP-dependent transcription of steroid hydroxylase genes in the adrenal cortex. *Journal of Biological Chemistry*, 269:27783-27783.

Wright, J. J., 2009. Diversity, phylogenetic distribution, and origins of venomous catfishes. *BMC evolutionary biology,* 9(1):282.

Yamazaki, F. and Donaldson, E. M., 1968. The spermiation of goldfish (*Carassius auratus*) as a bioassay for salmon (*Oncorhynchus tshawytscha*) gonadotropin. *General and comparative endocrinology,* 10(3): 383-391.

Yang, Z.W., Chen, J.P., Wang, T., Tian, Y., Liu, D.S., 2007. In vitro expression of *Lgeionella pneumophila* pilE gene and its immunogenicity. *Nan Fang Yi Ke Da Xue Xue Bao*. 27(2):141-5

Yaron, Z., 1995. Endocrine control of gametogenesis and spawning induction in the carp. *Aquaculture*, 129(1): 49-73.

Yu, H., Cheng, H., Guo, Y., Xia, L. and Zhou, R., 2003. Alternative splicing and differential expression of P450c17 (CYP17) in gonads during sex transformation in the rice field eel*. Biochemical and biophysical research communications*, 307(1): 165-171.

Zhou, L. Y., Wang, D. S., Kobayashi, T., Yano, A., Paul-Prasanth, B., Suzuki, A. and Nagahama, Y., (2007b). A novel type of P450c17 lacking the lyase activity is responsible for C21-steroid biosynthesis in the fish ovary and head kidney. *Endocrinology*, 148(9): 4282-4291.

Zhou, L. Y., Wang, D. S., Shibata, Y., Paul-Prasanth, B., Suzuki, A. and Nagahama, Y., (2007a). Characterization, expression and transcriptional regulation of P450c17-I and-II in the medaka, *Oryzias latipes. Biochemical and biophysical research communications*, 362(3): 619-625.

APPENDIX

Reagent Composition

1. Bacterial culture media and antibiotics

1.1. LB-Agar medium (Luria Bertani Agar Medium)

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

(Sterilized by autoclaving)

1.2. Ampicillin (100 mg/ml)

Ampicillin sodium salt	100 mg
DMW	600 µl
Ethyl alcohol	400 µl

(Used in the media at 100 µg/ml concentration)

2. Genomic DNA isolation

2.1. DNA extraction buffer

Tris-HCl	7.88g
NaCl	23.37g
EDTA	1.46g
dH_2O to	1000 ml

pH: adjust to 8.0

2.2. SDS (10%)

SDS	10 g
dH2O	90 ml

Volume raised to 100 ml.

2.3. Chloroform: Isoamyl alcohol (24:1)

Chloroform	48 ml
Isoamyl alcohol	2 ml

Stored at 4°C.

2.4. Sodium acetate (2M)

Sodium acetate	27.25g

Final volume made to 100 ml, pH adjusted to 4.0 by glacial acetic acid; Sterilized by autoclaving.

2.5. Tris-EDTA

1M Tris-Cl (pH 8.0)	1ml
0.5M EDTA,	200µl

Volume made up to 100ml.Sterilized by autoclaving; Stored at 4^{0}C.

2.6. RNase (10mg /ml)

RNase	10 mg
Sodium acetate (0.01M; pH 5.2)	1 ml

Heated to 100^{0}C for 15 minutes, 0.1 M Tris Cl (pH-7.4) 100 µl (for pH adjustment).

Dispensed in aliquots and Stored at -20^{0}C.

3. Preparation of recombinant DNA constructs

3.1. EDTA (0.5 M; pH 8.0)

EDTA	186.1 g
DMW	800 ml

pH adjusted to 8.0 with NaOH and sterilized by autoclaving.

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

Tris base	12.11g
dH2O	100 ml

pH adjusted to 8.0 with conc. HCl and sterilized by autoclaving.

3.3. TAE (50X)

Tris base	242 g
Glacial acetic acid	57.1 ml
EDTA (0.5 M; pH 8.0)	100 ml

Final volume made to 1000 ml; Sterilized by autoclaving.

3.4. Ethidium bromide (10 mg/ml)

Ethidium bromide	0.1 g
dH2O	10 ml

Covered with aluminium foil and stored at 4^{0}C.

3.5. Gel loading buffer (6X)

Bromophenol blue	0.25 g
Xylene cyanol	0.25 g

Glycerol	3 ml
DW	7 ml

3.6. Calcium chloride (0.1M)

CaCl2.6H2O	2.2g
dH2O	100ml

Sterilized by filtration through a 0.22 micron disposable filter; Stored in 1ml aliquots at 4^0C.

3.7. IPTG (0.8 M)

IPTG	2 g
dH2O	10 ml

Sterilized by filtration through a 0.22 micron disposable filter;
Stored at -20^0C.

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

X-gal	20 mg
Dimethyl formamide	1 ml

Stored at 4^oC.

4. SDS PAGE

4.1. SDS gel loading Buffer (2x)

1M Tris Cl(pH6.8)	500ul
10% SDS	2ml
Bromophenol blue	0.01g
Glycerol	1ml

Made up the solution to 5ml with dmw. Added 28.5ul Beta mercaptoethanol for 2ml of the 2x loading buffer just before use and used at 1x concentration for loading.

4.2. SDS PAGE Resolving gel buffer(1.5 M Tris-Cl, pH:8.8)

Tris base	18.2g
DW	80ml

Adjusted pH to 8.8 with 1N HCl and made volume upto 100ml using DW.

4.3. SDS PAGE Stacking gel buffer(1 M Tris-Cl, pH: 6.8)

Tris base	6.1g
DW	80ml

Adjust pH to 6.8 with 1N HCl and made volume upto 100ml using DW.

4.4. SDS PAGE Resolving gel(12%, 10 ml)

Distilled Water	4.6 ml
30% Acrylamide-Bisacrylamide (29:1) Mix	10.0 ml
1.5 M Tris-Cl(pH 8.8)	5.0 ml
10% SDS	0.2 ml
10% APS	0.2 ml
TEMED	0.008 ml

4.5. SDS PAGE Stacking gel(5%, 5 ml)

Distilled Water	3.4 ml
30% Acrylamide-Bisacrylamide (29:1) Mix	0.83 ml
1 M Tris-Cl(pH 6.8)	0.63 ml
10% SDS	0.05 ml
10% APS	0.05 ml
TEMED	0.005 ml

4.6. SDS Page Electrophoresis buffer (5X)

Tris base	15.1g
Glycine	94g
10% SDS	50ml

Make up volume to 1000 ml with DW.

4.7. Protein staining Solution(100ml)

Coomassie Blue R250	200mg
Methanol	50ml
Glacial Acetic Acid	7ml
DW	43 ml

Solution was mixed and filtered by Whatman filter paper to remove suspended particles.

4.8. Destaining solution 100ml

Methanol	30ml
Glacial Acetic Acid	7ml
DW	63ml

5. Western Blotting

5.1. Western Blotting Buffer

Tris base	3.03g
Glycine	14.4g

Methanol	100ml

Made up the volume to 1000ml using DW.

5.2. Phosphate Buffered Saline (PBS)

Nacl	8 g
KCl	0.2 g
Na_2HPO_4	1.44 g
KH_2PO_4	0.24 g
DW	900 ml

Adjusted pH to 7.4 using conc. HCl, and volume made upto 1000ml using DW.

5.3. Blocking buffer(3% BSA in PBS)

BSA	1.5g
PBS (pH 7.4)	48.5 ml

5.4. PBS -Tween 20(0.05%)

Tween-20	50ul
PBS (pH 7.4)	100ml

5.5. Horse Radish Peroxidase Substrate Solution

3,3'-Diaminobenzidine (DAB)	5-10mg
PBS	10ml

Add 10 µl H_2O_2 just before use.

6. Miscellaneous

6.1. DEPC water

DEPC	1ml
Sterile dH2O	1 L

ABBREVIATIONS

Δ	Delta
µg	Microgram(10^{-6}g)
µl	Microlitre(10^{-6}l)
µM	Micro molar
^{o}C	Degree centigrade
Amp	Ampicillin
bp	Base pair
BSA	Bovine serum albumin
Conc.	Concentration
Cm	Centimeter
CMV	CytomegaloVirus
Da	Dalton
DW	Distilled water
DNA	Deoxyribonucleic acid
DNAase	Deoxyribonuclease
dNTP	Doxynucleotide triphosphate
EDTA	Ethylene diamine tetra acetic acid
FAO	Food and Agricultural Organisation
Fig.	Figure
hr	Hour(s)
IPTG	Isopropyl-thiogalactopyranoside
Kb	Kilobases
KDa	Kilodalton
Lit	Litre
LB	Luria-bertani
M	Molar
Mg	Milligram(10^{-3} g)
Min	Minutes
Ml	Milliliter(10^{-3} L)
mM	Milli molar
Mol	Mole
mRNA	Messenger RNA
MW	Molecular weight
N	Normal
Ng	Nano gram
Nm	Nanometer
O/N	Overnight
PCR	Polymerase chain reaction
ppt	Parts per thousand
RE	Restriction Enzyme
RNA	Ribonucleic Acid
Rpm	Revolution per minute
S	Seconds
SDS	Sodium dodecyl sulphate
TAE	Tris acetate EDTA
TE	Tris-EDTA buffer
U	Unit

UTR	Untranslated region
V	Volt
X-gal	5-bromo-4-chloro-3-indolyl-b galactopyranoside
BLAST	Basic Local Alignment Search Tool
cDNA	Complementary DNA
rRNA	Ribosomal RNA
R.T.	Room temperature
UV	Ultra Violet