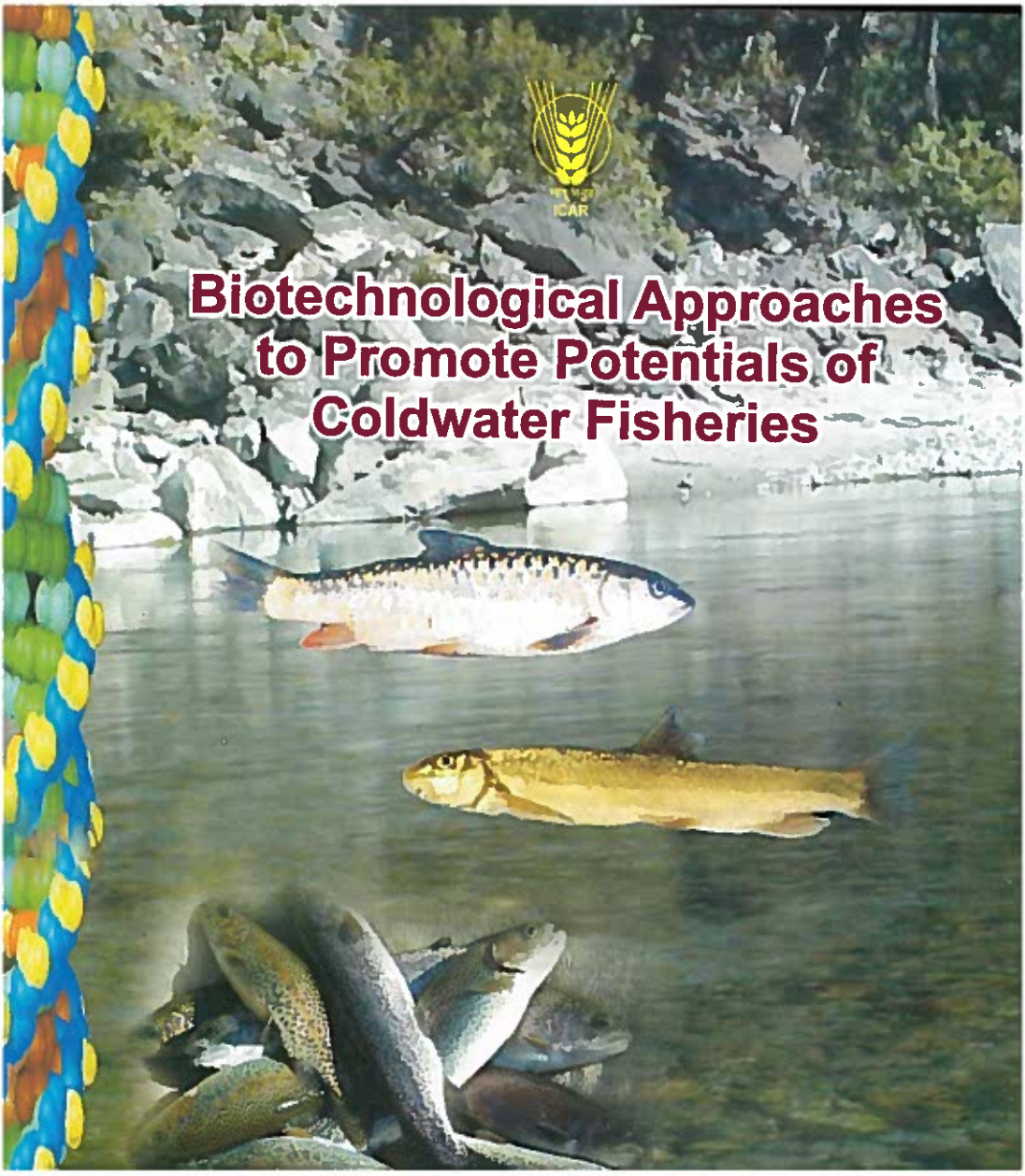

ICAR
Biotechnological Approaches
to Promote Potentials of
Coldwater Fisheries

भाकृ अनुप
ICAR

Biotechnological Approaches to Promote Potentials of Coldwater Fisheries

Compiled and edited by

Dr. Rajeev Kapila

Concept and Guidance

Dr. P.C. Mahanta

Bulletin No. 10 **2007**

NRC CWF
BHIMTAL

Biotechnological Approaches to Promote Potentials of Coldwater Fisheries

Published by : Dr. P.C. Mahanta
The Director, NRCCWF, Bhimtal (Nainital), Uttarakhand

डॉ. एस. अय्यप्पन
उप महानिदेशक (मत्स्य)
Dr. S. Ayyappan
Deputy Director General (Fisheries)

भारतीय कृषि अनुसंधान परिषद्
कृषि अनुसंधान भवन-II
पूसा, नई दिल्ली 110 012
INDIAN COUNCIL OF AGRICULTURAL RESEARCH
KRISHI ANUSANDHAN BHAVAN-II
PUSA, NEW DELHI 110 012

FOREWORD

The tools of biotechnology, apart from providing important insight into various biological process, are now being utilized for practical applications in medicine, agriculture, veterinary and industry. Understanding of critical biological process of fish at molecular levels has provided basic scientific inputs upon which modern aquaculture are based. Biotechnology mainly pertains to tools and techniques going across different disciplines. It would not only entail interactions and discussions with researchers in those areas but also trained man power. There is no doubt that biotechnology will have to play a key role in meeting ever increasing demands of fish production of the country.

The research needs in the fields of reproduction, genetics, pathology have immediate relevance to aquaculture especially in the inland fisheries in which coldwater fisheries is one of the important sub sector. Biotechnological interventions to solve various bottlenecks in hill fisheries may prove to be very relevant and interesting. Though, molecular approaches are still in the infancy stage in the fisheries sector as compared to other fields of agriculture but have lot of hidden treasure for best utilization of countries aquatic resources. Thus genetic engineering and biotechnological tools has gaind enormous importance in increasing fish production and biodiversity conservation in recent years. It is in the context the NRCCWF has initiated to take up investigations on the fish species which are very important in our Himalayan regions. The Institute has compiled the existing information in this important and frontier field of science and also presented the information generated on coldwater

PREFACE

The hill fisheries require different technology approach and support services because of entirely different ecological conditions from the rest of fisheries sector. The physiological adaptations of fish being poikilotherms are quite interesting. The fish in coldwater has to survive against many odd conditions like fast current of hill stream water and to chilled snow melted water. These fishes have developed beautiful mechanisms to cope up these adverse conditions. Only, recently the National Research Centre on Coldwater Fisheries apart from concentrating its efforts in developing technologies for framing of important indigenous fish species inhabiting Himalayan uplands has initiated looking into various physiological processes at molecular level using biotechnological approaches. There are many bottlenecks related to slow growth, breeding under captivity, low fecundity, loss of valuable germplasm due to inbreeding etc of coldwater fishes which create major constraint in exploiting full potentials of this virgin sector of fisheries. In recent, biotechnological tools have been proved to be effective in increasing productivity of agriculture and animal products. Hence efforts are needed to work out basic strategies using these modren tools for developing a complete productive system in temperate aquaculture.

The present document apart from reflecting the work done in this field of science with respect to fisheries, highlights the work done by NRCCWF in this important area. The information presented is based on the sustained efforts of our scientists involved in such projects time to time. All the workers of the institute who contributed in preparation of this document deserve appreciation. I am hopeful that

CONTENTS

Biotechnological Approaches to Promote Potentials of Coldwater Fisheries

1. Introduction

Aquaculture biotechnology is an enabling technology which not only feeds into the fish culture and aquatic plant farming industries but also their associated processing industries. Aquatic biotechnology includes several components: aquaculture biotechnology (fish health and brood stock optimization); aquatic bio-processing (obtaining valuable compounds from aquatic organisms); and bioremediation. The short term opportunities are primarily in aquaculture biotechnology applied to help in meeting the protein requirements of a growing world population while long term opportunities are expected in the form of pharmaceuticals, bioremediation and biosensors.

Aquaculture is predicted to continue playing a major and ever increasing role in meeting human needs for protein. The aquatic germ plasm resources are diverse and constitute an important component of food security. The fishery development in India since independence was mainly focused towards the capture, culture and processing of fin/shell fish stocks in warm-waters of inland and coastal regions of the country. The increase in production has been possible from aquaculture by employing better farm management practices, availability of desired seed and supplementary diet but nobody looked into the improvement of stocks to achieve higher yields, isolation of

The coldwater fisheries research in the country received serious attention after the establishment of an independent National Research Centre in 1988 located in Kumaon (Uttaranchal). Thus in last two decades of research in this area, the attention remained on the fish biodiversity, habitat analysis, ecology, biology and aquaculture of selected species and on development of significant breeding technologies. It was during the terminal year of IX plan and the first year of X plan that some efforts on biotechnology through biochemical investigations have been made. However, due to lack of expertise in the field and desired laboratories the progress has been slow. But institute has made some beginning thorough some projects in past years to set the process in motion. Though there are multiple applications of biotechnology in fisheries from production and culture to processing but few prospective applications relevant to coldwater fisheries under present scenario with research trends in other laboratories of world are discussed hereunder along with some of the results obtained at our institute's laboratories.

2. Transgenic and genes of interest

Over the last decade rapid progress has been made in the application of gene manipulation technology in aquaculture due to known biology of the organisms and large scale standardized farming techniques. For most of the species concerned, eggs are freely available in bulk, can be fertilized under controlled conditions, and do not require a return to the female reproductive tract for the completion of development (as is the case with mammals). Now, the availability of cloned gene sequences from many species and recently from finfish libraries it self, provides tremendous opportunities to genetically

are the first to initiate research in transgenic fish and Asia is the centre of research activity in transgenic fish. In India only few labs are involved in this work. Transgenic fish may play an important role in increasing production of coldwater sector but major constraint with indigenous fish species is that their breeding technologies under pond conditions are still in infant stage which is restricted to laboratories only. Maintenance of brooders and their maturation under captivity is a challenging task. However, transgenic with following genes may give a promising direction to the coldwater sector.

2.1 *Growth Hormone (somatotropin) genes*

Growth hormone is a polypeptide hormone synthesized in the anterior portion of the pituitary glands of all vertebrates and released into circulation and exerts stimulating influences over growth and development. Being a protein rather than a steroid hormone, it is broken down in the gut if fed to animals, at least in most vertebrates, although in young stages of some fish species it pass through the gut and exert influence via the dietary route. Like other polypeptide hormones, such as insulin, it is effective when injected, and it has been demonstrated that avian and mammalian growth hormones is effective in salmonid fish following injection. In most of the work done, mammalian growth hormone genes has been spliced to a mammalian metallothione promoters before insertion into fish eggs. This gene construct was used in the hope that the gene would be expressed continuously at relatively high frequency by liver cells, rather than intermittently and at low frequency by the pituitary gland cells. It is more advantageous to use cloned piscine growth hormone gene rather than using a growth hormone gene from mammalian source.

2.2 *Metallothionein genes*

Metallothioneins are proteins that bind heavy metals in cells, particularly cadmium, copper, zinc, and mercury. They have a dual function in the animal cell. The primary function is to supply cofactor to enzymes within the cell. Whereas, another key function is detoxification, a process which involves binding of these heavy metals within the cell by the protein, followed by excretion of the metallothioneons protein complex from the cell and the organism via the kidney. Metallothioneins are also inducible proteins, and their synthesis is greatly accelerated in the presence of heavy metals. Thus fish and other animals, when exposed to a sub lethal dose of a heavy metal such as cadmium or copper, are able to protect themselves by increased metallothionein synthesis. Now metallothionin have become best known in gene manipulation for their promoter sequence but these are interesting genes in their own right since it seems possible that fish or other aquatic animals, with increased numbers of these gene sequences, could better survive temporary heavy metal pollution. Increased population density and other constructional activities in the hills have led to increased rate of pollution in hill streams and lakes. Such transgenic fishes developed with detoxification genes like metaloothionin may prove to be boon in future.

2.3 *Antifreeze genes*

A number of species of fish living in Arctic or Antartic seas have evolved proteins in their blood which reduce the effective freezing point of the blood and tissues so act as natural antifreezes. One fish with such proteins is the winter flounder (*Pseudopleuronectes americanus*). The appropriate genomic sequence has now been isolated

gene transfer. The structural and functional features of antifreeze proteins (AFPs) enable them to protect living organisms by depressing freezing temperatures, modifying or suppressing ice crystal growth, inhibiting ice recrystallization, and protecting cell membranes from cold-induced damage. The versatility of the AFPs suggests that their production and commercialization would be a potentially profitable venture. AFPs and their genes can be used in fish and plants to enhance resistance to freezing. AFPs can be used in medicine to improve the cold protection of blood platelets (to extend their shelf life prior to transfusion); paradoxically, when used in conjunction with cryosurgery, they can help destroy malignant tumors. Their ability to inhibit recrystallization can improve the quality of frozen foods. In addition, antifreeze gene promoters are uniquely suited to drive the expression of functional genes, such as growth hormone that results in enhanced growth rates of salmonids (e.g., salmon, trout) and other fish species valuable to aquaculture. Recently attempts have been initiated at Institute's laboratory to amplify antifreeze gene sequence from *Schizothorax richardsonii* using polymerase chain reaction. The primers for amplification were designed taking gene sequence of anti-freeze gene of (685bp) winter flounder (figure 1) as reference.

tgaaacttcc tgatgatctg gtgacacctg ctggttgaag gaaacagagt
ttgagaggca gcagaaaaaa ttatttttagt ttaaatgaag aagctgtcat
ttgattttat gttgggggggg ggtcatcaca cacagatatt gataactgtc
atcactgagt ttggtgaaag tgacggacca gtaaatgttg tgatatataa
tattatcata ataattataa taataccatt aatctctgca gaatcactga
agccagcccc gaccccgcag ccaaagccgc cccagcagca gctgccgccc
ctgccgcagc cgccccagac accgcctctg acgccgccgc tgcagccgcc
cttaccgccg ccaacgccaa agccgctgcc gaactcactg ccgacaacgc
cgccgccgct gcagcagcca ccgccagagg ttaaggatcg tggtcgtctt

2.4 *Esterase genes*

Esterases are protein enzymes which hydrolyze esters. In insect it has been found that mosquitoes which have become resistant to some insecticides have done so by natural amplification of their esterase gene copy number and have thus been able to break down the insecticide more efficiently. It is conceivable that situations could arise where it would be an advantage to confer on fish an enhanced resistance to insecticide-like compounds. Large-scale intensive agriculture, especially tea cultivation has increased load of pesticides in mountainous water bodies. Large quantities of many types of organophosphorous compounds, weedicides and fungicides etc are used in tea growing hill states. The literature states that over 300 species of arthropods, 58 species of fungi and 130 species of plants infest tea cultivation in India alone, necessitating the continuous application of heavy doses of pesticides. And, despite the international markets' concern on the human health hazards posed by the toxic residues in tea, not less than 30 different pesticides are used. Estimates suggest that around 4,00,000 ha is under tea cultivation in India. Hill streams flow through intensive tea cultivation in many parts. These are perennial and create extensive grassy swamps in the uncultivated valleys during the rains. It can be assumed that any chemical run-off from the tea cultivation should automatically reach the swamps, affecting all aquatic life within the habitat. Under such conditions development of transgenic fishes with multiple genes of esterase enzyme which can breakdown pesticides to less harmful metabolites will be future's most desired commodity.

2.5 *Disease resistance genes*

2.6 *Regulatory gene sequences*

All genes are, at least in part, under the control of other DNA sequences. All eukaryotic genes have promoter sequences upstream from transcription start site, and many also have specific enhancer sequences luring further upstream or downstream. Promoter sequences are necessary for proper transcription to take place and often enhancer sequences regulate specific rate control in particular tissues. Some promoter sequences are themselves activated by specific factors or molecules such as steroid hormones, heavy metals, or heat shock. Since particular gene can be spliced to novel promoters there is considerable scope for beneficial gene manipulation in the production and injection into organisms of such potentiality fruitful combinations of regulatory sequences and coding sequences. Metallothionein gene promoter is an example of universal promoter. It is active in most tissues but particular active in liver cells. Cold/ heat shock gene promoters are examples of inducible promoters and can be induced by temperature change. All organisms have such genes and the sequence which responds to temperature changes are the upstream promoters. Such promoters sequences could prove useful if it were desirable to have trans gene which would only be synthesized on specific stimulation of organism by temperature.

3. Molecular genetics

Genetics and biotechnology have played a pivotal role in enhancing agriculture and animal production. It is only recently that genetics has acquired an important status in fishery science. Genetic improvement is the process of replacing a given population of genotypes with another that subtends superior phenotypic performance. Selection, by imposing differential reproduction

3.1 *Sources of genetic variability*

Genetic variation in a population is measured by the heterozygosity or the degree of polymorphism. There are two basic types of variability: recombinational and mutational. As is implied by its name, the recombinational variability consists of different combinations of existing alleles in the descendent population. The result of this variability is the enormous diversity of individuals capable of sexual reproduction. Random union of haploid gametes during fertilization produces a huge diversity of genotypes. A mutation happens when a pair of nucleotides is swapped by another, a pair (or a few of them) is dropped or added to a chromosome. These type of mutations happen during DNA replication, but a large number of them is immediately corrected by proteins involved in the process. Mutations are caused by different external factors, and can then be very frequent. Changes in DNA leading to mutations can be caused, for example, by X-rays, ultraviolet light (UV), or radiation occurring during decay of radioactive elements. Some chemical compounds like insecticides, preservatives or pharmaceuticals are known to cause mutations. Frequency of spontaneous mutations ranges from one in 100 thousand to one in a million copies of a single gene. A mutation can have no effect on the organism (a neutral mutation). If, however, it influences a change in the encoded protein or its functions, the mutation is usually harmful. Mutations are the main source of gene alleles. That is why they form a basis of evolutionary changes.

3.2 *Loss of genetic variability*

The loss of genetic diversity occurs due to the crash of the size of a population to a very small number of individuals due to habitat

generations. In a large, random breeding population, it is likely that an individual will be able to successfully find a mate who is almost completely unrelated to him or herself. However, in a very small population with a restricted range due to habitat fragmentation, the likelihood that an individual will mate with another who is completely unrelated decreases dramatically. In a normal population, there will be a low frequency of deleterious alleles present which are maintained in the group's gene pool because they are in the heterozygous state and their deleterious effect is masked by the dominant allele. When inbreeding occurs, the probability that two deleterious alleles will come together in one individual increases. This results in a larger chance that the deleterious effects of these recessive alleles will be 'seen' by natural selection. As this occurs, the average fitness of the population will drop as a consequence of a higher mortality rate, lower fecundity and a lesser ability to survive throughout the life stages. This loss of genetic variability due to habitat destruction, mass killing of fish and inbreeding of remaining brooders are the major cause that golden mahseer (*Tor putitora*), which was angler's delight in Himalayan lakes have come under the category of threatened fishes. Survey data of institute clearly depicts that the average size of snow trout fisheries has also decreased remarkably in recent times. This also points towards decreasing genetic variability of species. For developing suitable breeding programmes for better growth of coldwater fishes it's high time to initiate work for assessing genetic variability of stocks of indigenous species of coldwater with molecular tools.

3.3 *Techniques to assess genetic variability*

3.3.1 *Indirect Visualization of Genetic Variability*

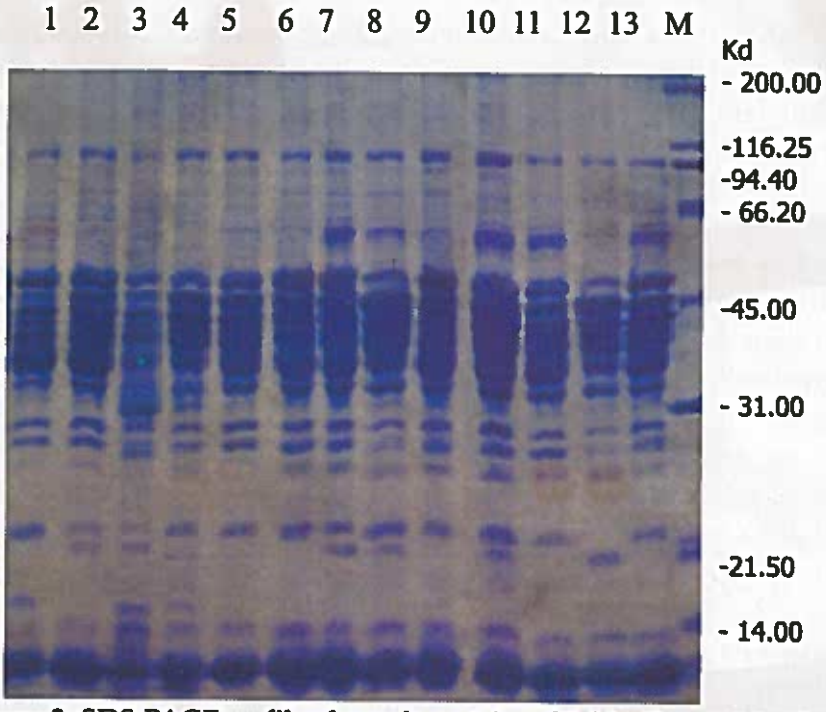
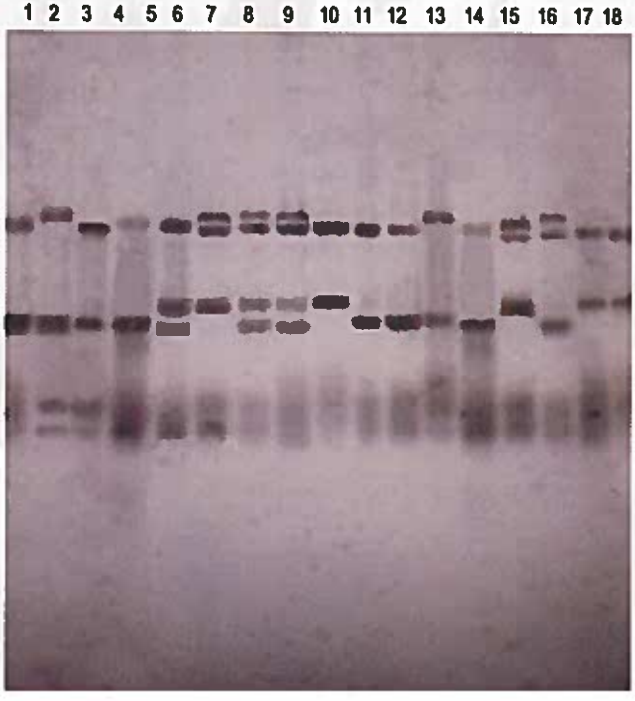


Figure 2: SDS-PAGE profile of muscle proteins of *Schizothorax richardsonii* (Gray)

Nowadays staining of specific enzymes on the gels are preferred over general proteins as genetic markers of fish stocks due to several reasons. First, the often complex patterns resulting from large number of bands revealed by nonspecific staining can make the interpretation of gels extremely difficult. Secondly, there is little or no supportive information available for interpreting any variation in banding patterns. Important information such as inheritance data from other species, data concerning the subunit structures of the proteins involved

Four populations of coldwater fish *Schizothorax richardsonii* (Gray) were analyzed using eight enzymes viz. Esterases, Malate dehydrogenase, lactate dehydrogenase, Alkaline phosphatase, Aspartate amino transferase, Xanthine dehydrogenase, Aldehyde oxidase and Glucose-6 phosphate dehydrogenase on native polyacryamide gels at NRCCWF laboratory and thus fifteen enzyme coding loci were screened. Five of these enzyme loci were determined to be polymorphic. Dendrograms prepared on these results revealed that the fish populations from neighboring rivers were much closer genetically than the distant ones.



polymophism (AFLP), Randomly Amplified Polymorphic DNA Fingerprinting (RAPD) etc. Each technique has its own advantages and limitations. Hence best option is to use various methods in combination instead of relying only one method. For example mitochondrial DNA fingerprinting shows maternal inheritance only. Mini-satellites fingerprinting approach reveals large numbers of loci simultaneously, producing highly variable and complex patterns in which individual locus genotypes cannot be distinguished. This variability is strength in some applications, notably in studies of parentage and breeding systems, but a severe limitation when individual locus genotyping is required. In case of micosatellites single locus probes or primers currently have to be developed new for each species, or groups of clearly related species, and the development phase may take several months of skilled and expensive labour. Similarly, RAPD markers are less expensive, faster, requires a smaller amount of DNA (0.5 to 50 ng DNA), does not involve the use of radioisotopes and requires less skill to operate but suffer from major limitation of exhibiting dominant recessive inheritance instead of co-dominant inheritance.

Ninety-eight RAPD (Randomly amplified polymorphic DNA) loci were screened at NRCCWF laboratory using 15 primers. OPA-04, OPA-16 and OPF 05 primers exhibited the maximum amount of genetic variation within and between the populations of *Schizothorax richardsonii* . On the basis of above results dendrograms prepared revealed that the fish populations of neighbouring rivers (Gola and Kosi, Chirapani and ladhiya)in Kumaon Himalaya were closer to each other in their genetic structure than distant ones

3.4 Cytogenetics

Cytogenetic studies have been carried out in India since 1960s. About 200 fin and shellfish species are now known cytogenetically. The diploid number of chromosomes in both the important indigenous species of coldwater viz. *Tor spp.* and *Schizothorax spp.* have been worked out to be 100 and 98 respectively (Lakra *et. al.*, 1997). The sex chromosomes could not be distinguished in either of species. Karyological studies using Ag-NOR banding also established genetic polymorhism in the number of nucleolar organizer regions (NORs) in Kosi and Beas river systems (Barat *et al.*, 1997).

4. Commercially important fish proteins/enzymes

Recently scientists have focused their attention to exploit commercially important fish proteins and enzymes from aquatic environment. Many fishes in coldwater are herbivorous and omnivorous like *Schizothorax richardsonii* and *Tor putitora*. Large amount of activities of enzymes like cellulases, xylanases, amylases, proteases and chitinases have been estimated in the gut of these fishes

Enzyme (IU/min/mg Protein)	Before Experiment	After Experiment	Fold Increase
Amylase	19.96 ± 4.12	24.44 ± 5.76	1.22
Protease	2.3 ± 0.34	2.68 ± 0.79	1.16

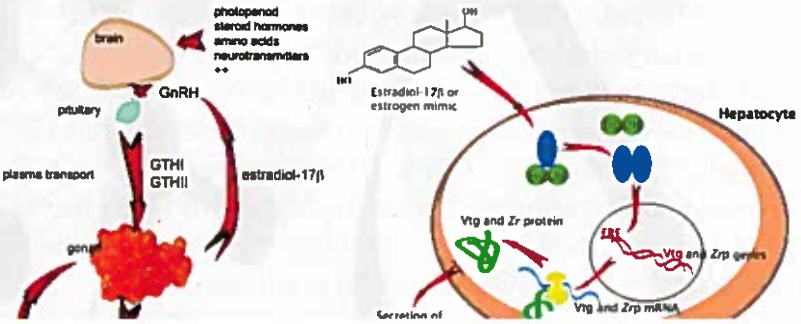
Figure 5 : Changes in digestive enzyme levels in *Tor putitora* fed with NRCCWF-III under field condition

5. Reproductive biotechnology

5.1 Gonadal maturation

A common principle for all fish is the production of large yolky eggs through the development of the oocyte. The formation, development and maturation of the female gamete and ovum (oogenesis) are intricate processes that require hormonal co-ordination. Oocyte growth is normally divided into four main stages, primary growth, and formation of cortical alveoli, the vitellogenic period, and final maturation. Oocytes are female ovarian cells that go through meiosis to become eggs. They are derived from oogonia, mitotic cells that develop from primordial germ cells migrating into the ovary early in embryogenesis. In teleost fishes, full-grown post-vitellogenic oocytes in the ovary are physiologically arrested at the G2/M border in first meiotic prophase and cannot be fertilized. In order for fertilization to occur, the oocytes must complete the first meiotic division and full-grown oocytes will resume their first meiotic division under appropriate hormonal stimulation. First meiotic division

regulate oocyte growth and maturation in teleosts and other vertebrates. Environmental changes, such as water temperature and photoperiod provide the cues to the central nervous system that triggers the maturation processes. In response, the hypothalamus secretes gonadotropin-releasing hormone (GnRH). As the central regulator of hormonal cascades, GnRH stimulates the release of GtHs from the pituitary. Although several GtHs have been identified from the teleost brain extract, two GtHs (GtH I & II) structurally similar to human follicle-stimulating hormone (FSH) and luteinising hormone (LH), respectively, are secreted from the teleost brain. GtH I (FSH) is involved in vitellogenesis and zonagenesis, while GtH II (LH) plays a role in final oocyte maturation and ovulation. GtH secretion is regulated through a feedback mechanism by estradiol-17α (E_2) and testosterone. Several feedback mechanisms also act on the gonadal development through the hypothalamus-pituitary-gonadal-liver axis (Fig 6A), because these organs produce substances influencing each other, leading to gonadal development and spawning. GnRH release is inhibited by dopamine, which in turn is affected by steroid levels. E_2



stimulates the production of Vtg and eggshell *Zr*-protein by the liver of female fish. The oocyte is the starting point for a new generation. Most of the machinery for DNA and protein synthesis needed for the developing embryo is made autonomously by the fertilized oocyte. However, in fish and in many other oviparous vertebrates, the major constituents of the egg, i.e. yolk and eggshell proteins, are synthesized in the liver and transported to the oocyte for uptake. Vitellogenesis, the process of yolk protein (vitellogenin) synthesis, transport, and uptake into the oocyte, and zonagenesis, the synthesis of eggshell *zona radiata* proteins, their transport and deposition by the maturing oocyte, are important aspects of oogenesis. The many molecular events involved in these processes require tight, coordinated regulation that is under strict endocrine control, with the female sex steroid hormone estradiol-17â in a central role.

5.3 *Detection assays/ tools*

Depending on the target organ or tissue, a wide variety of assays involving biotechnological tools have been developed to measure oogenic protein expression in fish. These include radioimmunoassays; enzyme-linked immunosorbent assays (ELISAs) and immunohistochemistry using monoclonal and polyclonal antibodies (Abs), RNA protection assay and transcript analysis by Northern blotting or various variants of polymerase chain reaction (PCR). Recently, the use of real-time (quantitative) PCR is increasingly becoming a valuable tool in oogenic protein analysis. In plasma samples, these assays vary in their sensitivity, but some have the ability to detect very low levels of protein expression, i.e. 1 ng/ml or less. RIA techniques are well developed for

has also been demonstrated using immunohistochemical analysis of exposed fish with specific antibodies. Immunohistochemistry is a valuable tool in the studies of estrogen and estrogen mimicking compound induced hepatic synthesis of Vtg and Zrp in oviparous vertebrates, especially in situations where blood samples are difficult to collect, e.g. when studying small-sized species.

5.4 Reproductive biotechnology in coldwater fishes

5.4.1 Hormonal manipulations

Use of biotechnological tools in fish reproduction of coldwater fisheries is the most needed areas for investigations. The chemical nature of stimulus needed by coldwater fishes particularly indigenous species has never been worked out. Maturation of *Tor putitora and Schizothorax spp* in pond conditions are still questionable and highly debatable issues. Only recently some work has been initiated at NRCCWF to quantify vitellogenin level in the fish reared in pond conditions for *Tor putitora* using polyclonal antibodies raised against common carp vitellogenin. This will give insight to the synthesis of vitellogenin in liver and its transport to gonads during breeding season and may delineate the cause of non maturation of fishes in pond condition. A successful attempt of induced breeding of golden mahseer (*Tor putitora*) was also carried out at Mahseer seed production unit, Bhimtal (Joshi,1988). The females were administered the preliminary doze of fish pituitary extract @ 6mg/kg body weight followed by booster doze of 8mg/kg body weight. Fertilization was carried out after 24 hrs of the first injection and fertilization rate achieved ranged from 34 – 92%. Similarly, preliminary trials without any major success

have also been observed to have significant impact on sex ratio in fishes. Moreover, golden mahseer in Kumaon waters have indicated large deviation from Mendelian sex ratio, which is 1:1. The males mature earlier than females and even their size and growth is comparatively significantly different. Experiments were also conducted to visualize the impact of different thermal regimes and photoperiodicity (Day length and artificial lights) on sex ratio of this priced fish of Himalaya. The role of melatonin, a neuropeptide secreated by pineal gland of fish in response to various environmental stimuli on sex ratio of mahseer was also ascertained under this programme.

5.4.2 Cryopreservation of fish gametes

The fish species can be protected, conserved and propagated in their natural habitat by forming fish sanctuaries or developing live gene banks. The fish germplasm may also be conserved and protected in form of frozen gametes and embryos. Cryobanks can play a crucial role in the genetic management and conservation of aquatic resources. The introduction of cryopreservation technique in fishery sector for development of gene/sperm bank has opened up new avenue for ex-situ conservation of germ plasm and development of fishery by improving quality seed production in hatcheries using cryopreserved milt. Genes from valuable individuals or stocks, which have desirable characteristics, can be preserved for future use and development. In such cases, representative genome in the form of sperms can be selected for cryopreservation. Cryobanks/sperm banks now days finding their increasing role in hatcheries for seed production. They have improved the efficiency of hatcheries and also reduced the cost of maintenance

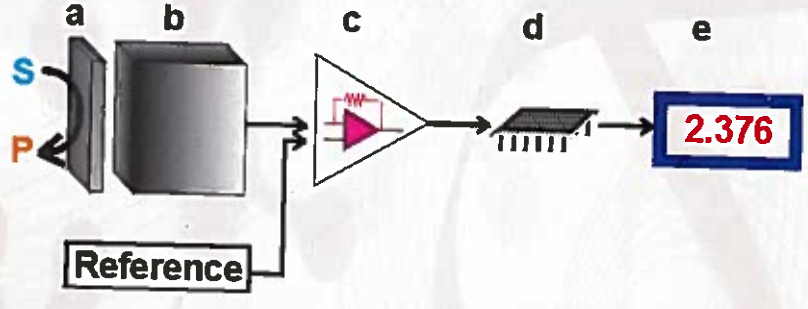
5.4.3 *Endocrine Disrupting Chemicals (EDCs)*

An endocrine disrupting chemical (EDC) may be defined as "an exogenous compound that causes adverse health effects in an intact organism, or its progeny, consequent to changes in endocrine function" . Estrogens mimic female hormones while androgens mimic male hormones. Thus estrogens exert a feminising effect while androgens exert a masculinising effect. They can be classified as agonists if they mimic a hormone or antagonists if they inhibit a hormone. EDCs comprise a diverse range of chemicals with various organizations differing slightly as to their selection of chemicals as EDCs; however the number of chemicals is over 100. The major groups comprise estrogens as well as synthetic analogues used in the contraceptive pill. Illicit drugs such as the anabolic steroids, some of which have legitimate uses, can also be included. Pesticides such as DDT and dieldrin also misbalance hormonal regulation of aquatic animals. Tributyltin used as an antifouling agent in boat paints is also an endocrine disruptor. Many of the effects caused by these substances have been associated with developmental, reproductive and other health problems in aquatic and laboratory animals. There is also growing concern that these compounds may be affecting humans in similar ways. The detailed mechanisms by which xenoestrogenic compounds mediate their induction of oogenic proteins is not fully understood, but it is known that they can bind with high affinity to the Estrogen receptors (ER) (as agonists) and initiate cell synthetic processes typical of natural estrogens. Some compounds also have the ability to bind to the receptor, but not eliciting estrogenic activities (as antiestrogens or antagonists), thereby blocking the binding site of natural estrogens. During ovarian recrudescence, incorporation of

xenoestrogens, toxicologists and biologists have used the induction of Vitellogenin (Vtg) and *Zr*-protein in male and juvenile oviparous vertebrates as an effective and sensitive biosensor for xenoestrogens.

6. Biosensors

A biosensor is an analytical device which converts a biological response into an electrical signal. The term 'biosensor' is often used to cover sensor devices used in order to determine the concentration of substances and other parameters of biological interest even where they do not utilise a biological system directly. Biosensors represent a rapidly expanding field, at the present time, with an estimated 60% annual growth rate; the major impetus coming from the health-care industry but with some pressure from other areas, such as food quality appraisal and environmental monitoring. Research and development in this field is wide and multidisciplinary, spanning biochemistry, bioreactor science, physical chemistry, electrochemistry, electronics and software engineering. Most of this current endeavour concerns potentiometric and amperometric biosensors and colorimetric paper enzyme strips.



is most often based on inhibition of the activity of selected enzymes such as cholinesterases, organophosphate hydrolase, alkaline and acid phosphatase, ascorbate oxidase, acetolactate synthase and aldehyde dehydrogenase. Enzymatic biosensors were developed using various electrochemical signal transducers, different methods of enzyme immobilization and various measuring methodologies.

6.2 Immunosensors

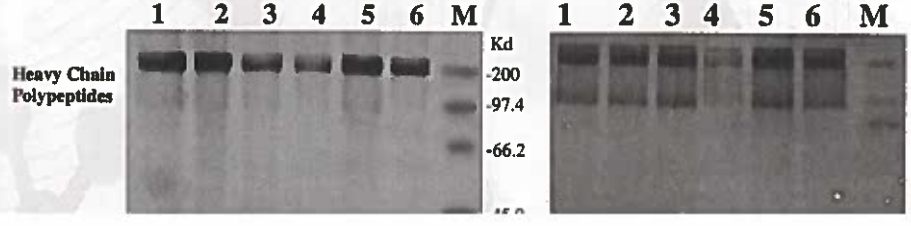
Biosensors may be used in conjunction with enzyme-linked immunosorbent assays (**ELISA**). ELISA is used to detect and amplify an antigen-antibody reaction; the amount of enzyme-linked antigen bound to the immobilized antibody being determined by the relative concentration of the free and conjugated antigen and quantified by the rate of enzymic reaction. Enzymes with high turnover numbers are used in order to achieve rapid response. The sensitivity of such assays may be further enhanced by utilizing enzyme-catalysed reactions which give intrinsically greater response; for instance, those giving rise to highly colored, fluorescent or bioluminescent products. Recently ELISA techniques have been combined with biosensors, to form **immunosensors**, in order to increase their range, speed and sensitivity.

Growing hill population and developmental activities and booming tourism industry has also increased the instances of endocrine disrupting substances in mountainous lakes, reservoirs and rivers. Developing such biosensors with can detect endocrine disrupting molecules with maximum sensitivity are the much needed areas of research for keeping whole biodiversity intact.

scale genomic sequencing projects and with throughput approaching the industrial scale a more or less complete catalogue of genes and their coded proteins are now available for an increasing number of selected organisms. This greatly facilitates the identification of genes and allows issues relating to genome organization, architecture, and evolution to be addressed. Equally significant are technical developments that allow gene expression at the level of transcript and its encoded protein to be conveniently addressed. This can be undertaken on a scale that matches the entire genome, allowing the expression profile of large numbers of genes and proteins to be characterized. Such profiling constitutes an open screen of genes and proteins, identifying those whose expression is regulated in relation to changes in performance of the whole system due to changes in environmental conditions in which the organism is harboring. In a recent study, a group of scientists using micro array technology identified 260 unique cDNAs that were significantly differentially expressed in various tissues of common carp. The majority (252) of transcripts increased in expression upon cooling, reflecting a basic paradigm of cold acclimation: that organism frequently compensate for the rate determining effects by synthesizing more enzymes to increase biochemical performance. It was observed that nucleic acid processing (40 genes), transport genes (37), protein catabolism (35), cell stress or molecular chaperones (21), metabolism 18, signaling (13) and cell structure (12) genes were up-regulated. Only eight genes were down regulated by cold adaptation.

7.2 *Classical biochemical studies*

little or no value in assessing the physiological status or performance of a system or of mechanisms mediating the response. There is no doubt that regulation of transcript is just one of several levels at which biological regulation occurs and that the phenotype results from integration at all levels. As a comparative example, the Δ^9 -desaturase gene is upregulated in many diverse organisms in response to cooling, and this is mediated, at least in part, by transcriptional up- regulation. However, the induction of the transcript tends to be short term and transient, whereas that of its encoded protein may be delayed and more long-lasting owing to the more extended life time of protein translational complex and slower turnover of protein protein may be delayed and more long lasting owing to the more extended life time of the protein translational complex. And slower turn over of protein relative to the transcript. Similarly in response to heat stress, the time course and expression level of heat shock protein transcript vary between closely related species and are linked to the levels of the synthesized heat shock protein transcripts vary between closely related species and are linked to the levels of the synthesized heat shock proteins. Thus responses linking transcript amount to physiological responses are likely to be more evident by measurements made during responses to changed circumstances or to physiological stimuli than in the ensuing steady state.



Recently studies were made to delineate the biochemical mechanism of cold tolerance in cold water fishes at NRCCWF laboratory. It was observed that the levels of glycolytic enzymes (pyruvate kinase and lactate dehydrogenase), enzymes pentose phosphate pathway (Glucose 6-phospate dehydrogenase), nerve transduction (Acetylcholine esterase) and muscle contraction (ATPase) increased many folds when fishes were exposed to cold temperature of 5^{0}C than when reared at 20^{0}C. New type of isoforms of proteins appeared in muscle tissues of coldwater fishes when get adapted to cold temperature as compared to higher temperatures (Fig. 8). These type of cellular adaptations make the coldwater fishes very distinct from warm water/ freshwater fishes in their various physiological responses to environment acclimatization. The variation in cellular responses to cold acclimatization are depicted in figure 9.

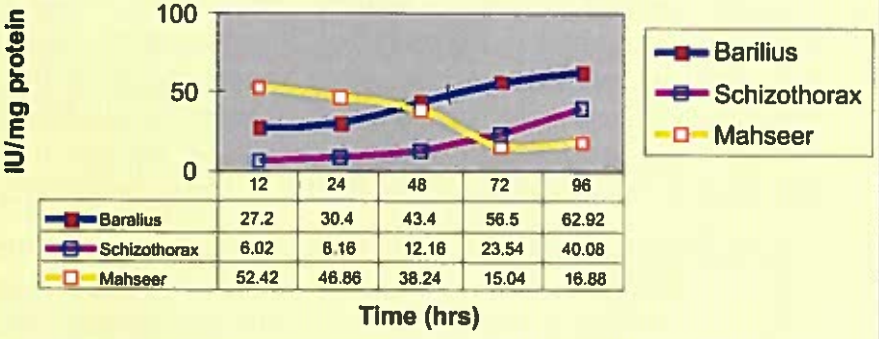
8. Future thrust areas

- Molecular genetic markers are basically of two types, protein and DNA. Moreover, all types of genetic markers used till date have some advantages or limitations. So judicial use of genetic markers depending upon the problem to be solved is the need of the day instead of wasting time and money on useless information.

- Atlas of all specis of coldwater fishes may be prepared linking with morphological characters using molecular taxonomic markers.

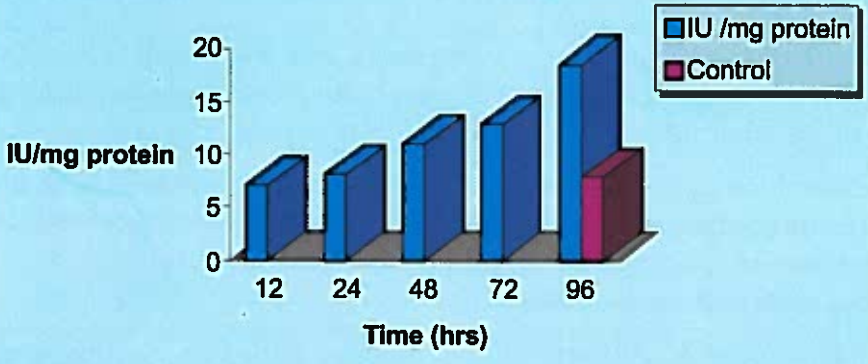
- Identification of genetic markers linked with commercially

Impact of Cold on Muscle ATPase of Coldwater fishes



	12	24	48	72	96
Baralius	27.2	30.4	43.4	56.5	62.92
Schizothorax	6.02	6.16	12.16	23.54	40.08
Mahseer	52.42	46.86	38.24	15.04	16.88

Impact of Cold on muscle G-6-P dehydrogenase of *S. richardsonii*



hard work and patience but these have potential to revolutionize the production of indigenous fishes of coldwater water.

- Genetic maps are being complied with two distinct goals: to provide resource for genetic analysis and to help dissect the evolution of genome organization by comparing linkage relationship for homologous genes. Mapping of genes of commercially important fishes has got potential use in locating loci affecting productive characters or disease susceptibility/ resistance for manipulation of these loci in breeding programmes.
- Use of biotechnological tools need not only specialized equipments but also trained manpower. There is a strong need of providing training as well as concept of basics of molecular biology before addressing any problem of fisheries. The course curriculum of fisheries graduates must be modified accordingly so that strong team of fish biotechnologist can be prepared.

9. Challenges

Challenges that are now being faced, and which will be faced in the foreseeable future, by the Fisheries biotechnology are parallel that to every other development made by biotechnology in other areas.

Research Support: Biotechnology is a knowledge-based activity. Attracting and retaining highly skilled and highly educated staff continues to be a concern. In addition, the research cycle is necessarily long, with high costs and risks.

Basic Research: Aquatic biotechnology, although having some impressive isolated success stories, is still embryonic. The tools

fisheries science, the equipment and other facilities are in serious need of modernization. Many laboratories do not meet standards and staff are not trained in the most current techniques. Eventually, the public's willingness to accept these new technologies will hinge on their acceptance not only of the product but the conditions under which it was developed and is maintained. It is an understatement to say that there are unique challenges posed by working in the aquatic environment (ensure physical and biological containment).

Intellectual Property Protection/Technology Transfer: Researchers and industry are concerned about protecting their intellectual property. World-wide progress on intellectual property rights is seen to be beneficial. Similarly, Challenges that are now being faced, and which will be faced in the foreseeable future, by the aquatic biotechnology industries parallel that to every other development from biotechnology.

Applied Research: There is a great need to develop regulations that will protect the environment and, at the same time, allow the use of modern biotechnology and its applications. To do this, research to determine potential environmental impacts, research on risk assessment, analysis and management methodologies, research on physical and biological containment techniques are all needed, to name but a few. Other applied research priorities include flexible mechanisms for technology transfer need to be better developed. And, cost recovery and cost sharing of expensive research done in government laboratories continues to be an issue. Although not unique to fisheries and aquaculture biotechnology, the inability to give proper job satisfaction and salaries to the highly educated and trained manpower resulting in an outflow of scientific and technical

assessments of transgenic fish and fish biologicals. Such applied research would lead to more effective regulations and create the necessary public confidence required for commercialization. The issue of public confidence and acceptance, not only of the product but of the way in which it is produced is becoming increasingly important in aquaculture and will be even more important in aquatic biotechnology. For better or worse, there is greater public concern over the aquatic environment than there is for terrestrial plant products. Escape, leading to changes in the genetic make up of wild stocks, is a major concern. For fish, the commercialization of transgenic brood stock is the biggest biotechnology issue (although maintaining such fish in secure facilities is not impossible).

The early development of the Indian aquaculture biotechnology industry depends in part on a strong Indian aquaculture industry on the whole. Without this strong receptor industry, the aquaculture biotechnology industry will not mature resulting in an outflow of the products, services and technology. For example, countries ability to commercialize gene probes and disease diagnostics, vaccines and antibody-based tests developed here are a concern because the receptor industry is smaller and has lower margins, making it more difficult for a product to have commercial success.

Literature Consulted

The author has consulted various reports, reviews, research papers and internet websites during the preparation of this manuscript in addition to his own work in this area.