FISHERIES, AQUACULTURE AND BIOTECHNOLOGY

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Preface

Now, mankind is in a state of transition from hunting and gathering to the production of fish and allies products with the advent of modern approaches and technologies of biotechnology and genetic engineering to traditional fisheries and aquaculture industry. Fisheries and aquaculture have been practiced since antiquity and the current revolution was brought about by economic, environmental and other technological realities. With the growing demand for fish and aquaculture products, biotechnology can help in the development of high quality, economic produce, thereby reducing dependence on natural populations. Moreover, rapid increase in global fisheries and aquaculture raise fears of continues environmental degradation of the aquatic, freshwater and marine environments.

Application of modern biotechnology to enhance fisheries and aquaculture is now most important for many reasons, particularly, those that are increasingly useful in practical situations of fisheries and aquaculture have led to their correct implementation for maximum benefits. The impact of biotechnology in fisheries and aquaculture represents a range of technologies to increase growth rate in farmed species, to improve nutrition of aquafeeds, to improve fish health, to help restore and protect environments, to extend the range of aquatic species and to improve management and conservation of wild stocks.

Until now, biotechnology has helped in the discovery and development of vaccines against enteric red mouth disease, vibriosis, furunculosis and gaffkemia. As the fisheries and aquaculture industry expands, the applications of biotechnology to produce fish vaccines and diagnostic kits for infectious diseases is keep increasing. Also, application of biotechnology to disease diagnostics has led to the discovery and development of fluorescent antibodies, enzyme linked immunoassay and the polymerase chain reaction (PCR) based very sensitive diagnostic kits. A combination of these newer technologies with traditional diagnostic approaches provides powerful means of controlling disease outbreaks.

The main concern of this book is to ultimately address the impact of modern biotechnology to fisheries and aquaculture. With fourteen chapters written by key eminent researchers of international and national organizations, Fisheries, Aquaculture and Biotechnology comprise overviews of the survey on the biology, ecology and genetics of Mexican oysters, role of autonomous vehicles in fisheries and aquaculture, bioengineering natural and artificial reefs, application of DNA fingerprints in the study of seaweeds, genes involved in the compensatory growth induced by refeeding, aquaculture exploitation, genomic analysis of Scamp and Yellowmouth grouper, detection of pathogenic bacteria, masculinization, status of shrimp farming in India, molecular response to stress, and diversity, distribution and bioprospecting of fish genetic resources in India.

This book is of immense theoretical and practical use to advance students and researchers of aquatic biology, freshwater biology, marine biology, fish biology, fisheries, aquaculture, population biology and of course biotechnology. This will be helpful as a vital reference to fish farmers, fisheries scientists, aquaculturists, industry developers, conservation biologists, environmental scientists, resource managers and policy makers and all those working in fisheries and aquaculture management and research through biotechnology.

We wish to thank Professor S.S. Purohit for his keen interest and continuous encouragement throughout the preparation of the book for publication. We also would like to extend our sincere thanks and great appreciation to those contributors from India, Italy, Japan, México, China, Spain and USA who took their real time to prepare such informative reviews for this book.

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1

A Survey on Biology, Ecology and Genetics of Mexican Oysters for Biotechnology Purposes

FAUSTINO RODRÍGUEZ ROMERO

1.1 INTRODUCTION

Crassostrea Sacco is the only valid genus in the Subfamily Crassostreinae (Lawrence, 1995). It is accepted that the species of this taxon, were derived of the members of the subfamily, Ostreinae during the Cretacic when the populations of these bivalves moved toward the coastal areas (Yonge, 1960).

The characters and tendencies of taxonomic value that define the species in this subfamily have been based in external morphology features. Gunter (1954), stated that within certain limits, defined by the fact that the plasticity in shell form has caused much confusion in oyster taxonomy, as many morphological variants of one species are similar to those of other. Indeed, the distinction of an oyster genus upon shell morphology alone has been questioned by a number of authors (Ranson, 1942; Gunter, 1950) as macroform is strongly influenced by substrate (Galtsoff, 1964; Palmer and Carriker, 1979). Other valuable approaches have been achieved by means of the use of other characters in their anatomy (Hirase, 1930; Nelson, 1938; Menzel, 1956; Castillo and García-Cubas, 1986) and of reproductive habits (Orton, 1928). To understand the evolutionary dynamics of the oyster species and their possible relationships with extinct taxa, approaches have been contributed by other disciplines besides the paleontology (Stenzel, 1971), such as the

cytotaxonomy (Longwell *et al.,* 1967; Menzel, 1968; Ahmed, 1973; Rodríguez-Romero *et al.,* 1978, 1979a, 1979b; Rodríguez-Romero, 1991; Thiriot-Quievreux and Insua, 1992; Ladrón de Guevara *et al.,* 1996), the biochemical genetics (Buroker *et al*, 1979a, 1979b, 1983; Guinez *et al.,* 1982; Hedgecock and Okazaki, 1984; De la Rosa and Rodríguez-Romero, 1988) and of the molecular biology (O Foighil *et al.,* 1998; Bierne *et al*, 1998; Hare *et al.,* 1996; Hare and Avise, 1998; Hrincevich *et al.,* 1995; Wakefield and Gaffney, 1996; McGoldrick and Hedgecock, 1996; Li and Hedgecock, 1996; Gaffney, 1996; Reeb and Avise, 1990).

The organisms of this genus are oviparous, whose life cycle is typical of the marine and estuarine invertebrate animals with a long period of larvae dispersion and a sedentary state in the adults. In *Crassostrea*, the left valve exceeds the size of the right valve in convexity; the umbo of the left valve is pronounced and significantly hanging; the previous ligament is developed and it is located far from all the precursors of the teeth. The mature oysters present a characteristic anatomical feature, the promial cavity. The scar of the abducent muscle is non orbicular (Lawrence, 1995). As a rule, oysters *Crassostrea* species do not stop growing after reaching certain proportions but continue to increase in all directions and consequently may attain considerable size (Galtsoff, 1964).

The marine and estuarine waters of the coasts of Mexico have acquired an importance as a source of food and for their potential for industrial purposes due to the diversity of exploitable plants and animals found there. From the fisheries point of view, some species resent already the effects of the excessive exploitation by the intensity with which are extracted. However, this circumstance has not motivated yet the use of modern technology in aquaculture for their better utilization in the short term. In the case of the Mexican oyster resources, the main production is obtained by exploitation of the wild populations and not even they have been tested fishing procedures technically efficient and even less a modern oyster culture with scientific bases and frontier methodology. On the other hand, the degradation of water bodies suitable for aquaculture of our coasts is every time acute due to the harmful effects that the pollution has caused as a consequence of the industrial activities, agriculture and urbanization in these regions. This is reflecting in the health of the aquatic environment and in the biological productivity of those habitats. Because of this, the exploitation on coastal waters should consider the value of the conservation of the renewable natural resources (Chew, 1993).

There are about one hundred oyster species in the world, many of those used in oyster culture. The species of *Crassostrea* of commercial

interest are found distributed in various parts the world. In Mexico, four autochthonous and an introduced species are suitable for commercialization by the feasibility of be exploited by means of their farming.

Evidences indicate that the Mexican commercial oysters species of the coasts of the Atlantic and of the Pacific share a common ancestry and that the interspecific differentiation in these organisms has been influenced by the environmental conditions where the populations have been developed. The adaptive processes and the isolation promoted by the continental displacement of the Central American bridge contributed to the slow independent evolution of the populations of oysters isolated since the plio-pleistocene. It is believed that the dynamics of the speciation and the relationships in these oysters could be visualized through multidisciplinary approaches included the genetics and the molecular biology. Due to their abundance and commercialization potential the main species in Mexico are: In the Atlantic, *Crassostrea virginica* Gmelin and *C. rhizophorae* Guilding and in the Pacific, *C. corteziensis* Hertlein. For the studies up to now carry out, an incipient speciation is suggested in these species that do not still impede the interspecific genetic flow. The essays of interspecific hybridization and the success in the viability of the hybrids among these three species demonstrate this close parental relationship and an interesting potential for the biotechnological manipulation of their genomes.

1.2 THE COMMERCIAL OYSTER RESOURCES OF MEXICO

In studies based on experimental capture with the purpose of fisheries exploration in the coasts of Mexico, it has been determined the presence of species of oysters belonging to the genera *Ostrea* and *Crassostrea*. The genus *Crassostrea* in this region contains four important species from the commercial point of view: *Crassostrea virginica* and *C. rhizophorae* in the coasts of the Gulf of Mexico and *C. corteziensis* and C. *iridescens*, along the coast of the Pacific; however, *C. virginica* is the one which registers greater percentage of capture (Figure 1.1). The historical record of the oysters production in the coasts of Mexico between 1986 and 1997 (SEMARNAP, 1998) indicates variations that by the moment are difficult to attribute to a cause in particular due to the existence of factors related to the capacity of capture and to the cyclical modifications of the world climate but a production average of 42,135.5 tons by year is observed;

although registered important low of production for the period 1992-1995 equivalent to the 24%, 38.7%, 13% and 24.1% respectively and an increase of the 33.2% and 34.3% for the period 1989-1990. For 1997, it slightly exceeded the historical average in a 1.9% (Table 1.1). The annual volume of the oyster production in disembarked weight for 1997 (SEMARNAP, 1998) in our country was 42,986 tons and the volume of the oyster production in live weight was 42,969 tons. The states that achieved greater production in disembarked weight as well as in live weight were: In the Pacific: Peninsula de Baja California 1,230 tons and Sonora 1,243 tons; while in the Gulf of Mexico, emphasize by their production: Tabasco with 17,893 tons and Veracruz with 14,285 tons respectively. The total of the oysters production in live weight included the production by cultivation, represents 2.74% of the yearly fisheries production; equivalent to the 1.05% of the nation annual fisheries products.

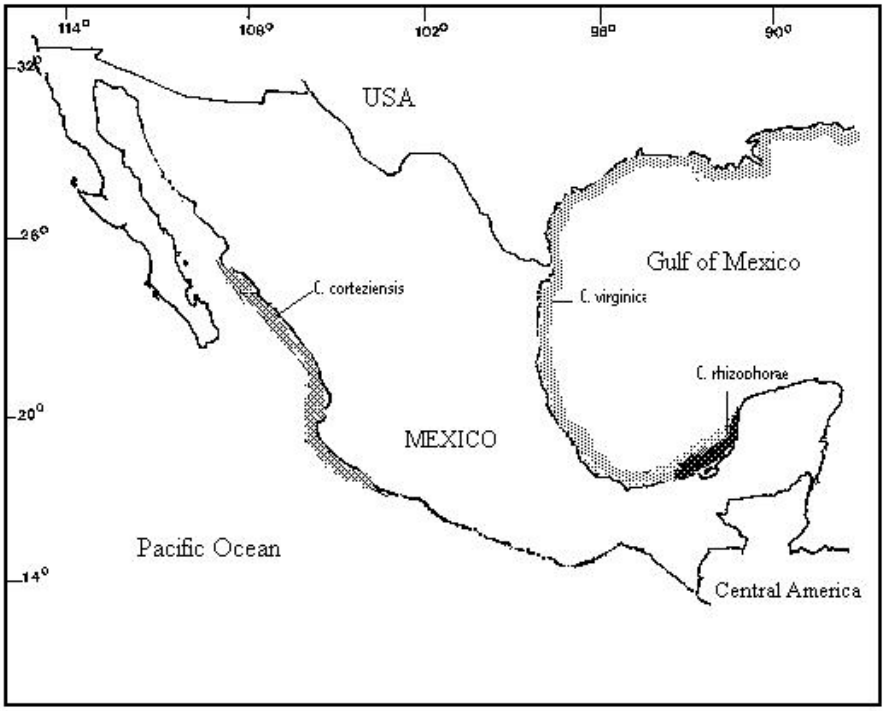

Figure 1.1: Geographic distribution of commercial Crassostrea species along the Mexican coast.

Table 1.1: Genetic similitude in Mexican populations of *Crassostrea virginica*

Locality	Geographic Position	Genetic Similitude
Laguna de Términos Campeche	18 65' N - 91 50' W	
Laguna de Mecoacán Tabasco	18 20' N - 93 08' W	0.989
Carmen-Machona Tabasco	18 19' N - 93 50' W	0.991
Sontecomapan Veracruz	18 32' N - 94 02' W	0.957
Laguna La Mancha Veracruz	19 33' N - 96 27' W	0.966
Laguna Tamiahua Veracruz	21 50' N - 97 33' W	0.959
Laguna Pueblo Viejo Veracruz	22 50' N - 97 53' W	0.941
Laguna Madre Tamaulipas	24 48' N - 94 50' W	0.908

1.3 OYSTER CULTURE IN MEXICO

In Latin America, the oyster culture constitutes a source of attractive income for countries as Brazil, Colombia, Chile, The United States and Venezuela. In Mexico, although the oyster culture has been practiced several years ago mainly in the Baja California coast, Sonora, Nayarit, Veracruz and Tabasco, the gross annual production is supported by wild and cultured oysters but official information is related only to the production obtained supposedly by oyster culture. For 1997, the national oyster production through cultivation was 40,381 tons. Of this, the coasts of the Mexican Pacific provided 2,910 tons alone (7.2%) while for the coasts of the Gulf of Mexico 37,471 tons (92.8%) were obtained. There is not reliable data on a historical series related to the oyster production by cultivation in the lapse from 1990 to 1997 that could indicate clearly the behavior of the annual production by this method. Nevertheless that in Mexico the cultivation of these bivalves is promising it is convenient to indicate a variety of conditions that limit the production of oyster by means of their culture. Attention should be paid to factors as water quality, salinity, climate, annual precipitation, annual temperature, global climate change; the biology and genetics of the organisms to be cultured, abundance and quality of the larvae because the oyster culture is based on wild spat collection and no selective breeding is practiced, biotic potentials of organisms at larval, juvenile and adult stages, conversion of food rates, resistance to diseases, nutritional habits, sexual cycles, taxonomic identification and management of the oysters for marketing. Concerning the available aquatic surface for the development of the oyster culture, the estimates on the potential of the country

indicates that currently is exploited no more than the 10% of the water bodies that auspicious the oysters cultivation.

1.4 TECHNOLOGY FOR THE MEXICAN OYSTER CULTURE

The advances of the modern technology for the oyster culture have permitted the design of cultivation systems with surprising production volumes. However, adequate conditions concerning the mentioned biological and environmental factors are indispensable for this. Unfortunately in Mexico, the quality of the water as well as the nourishment is not sufficiently controllable in these systems. In the case of the founder oysters that serve to begin the commercial cultivation in conditions of the oyster farming the risk exists for the success since genetic quality to resist diseases and to achieve better conversion rates of the food is not guaranteed. This has been the origin of investment failures due to parasitism; high mortality and undesirable low grow rates that make this activity expensive. That is why the Mexican oyster farms need genetically enabled oysters to guarantee the survival and good growth rate until their marketing.

1.5 GENETIC RESOURCES

The genetic research on populations of *C. virginica* by means of enzyme electrophoresis (Buroker, 1983) demonstrated a high genetic similarity near 99% between 18 studied populations and a sensitive separation with the population corresponding to Brownsville Texas, to the south of Corpus Christi where this value was calculated in 93%. This relative genetic difference in these populations has been attributed to the interruption of the natural genetic flow by the pattern of migration of the larvae and the presence of the Laguna Madre. For the case of the Mexican populations of *C. virginica* (De la Rosa and Rodriguez-Romero, 1988), the comparison among the population of oysters of the Laguna de Términos with populations along the Mexican coast of the Gulf of Mexico, a decline in the genetic similarity in function of the geographical altitude was evident; while in the south a high genetic similarity exists among the populations of oysters of Laguna de Términos, Mecoacan, and Sanchez-Magallanes, Tabasco, this similarity descends toward Tamiahua and Pueblo Viejo, Veracruz, reaching its extreme decline in the oyster banks of the Laguna Madre of Tamaulipas. These results have been

interpreted by these authors as a tendency to the independent evolution from the populations of oysters to both sides of the Laguna Madre and confirm that the populations with more attractive genetic variability are located in the north coast of Tamaulipas State. The studies on population genetics in *C. rhizophorae* and *C. corteziensis* are scarcer than in *C. virginica*; but it has been possible to determine that *C. rhizophorae* shares 72% of the same genes with *C. virginica* (Buroker *et al.,* 1979b). The results of Hedgecock and Okasaki (1984) in studies of variability and distance genetics among these three taxa have indicated that they are well defined as different species; while Hrincevich *et al.* (1995) were able to establish a molecular approach to discriminate *C. virginica* and *C. rhizophorae* by means of the analysis of the variation of the nucleotide sequence of the mitochondrial cytochrome oxidase I gene.

Speciation and accurate identity of species of these organisms is of considerable interest for biologists and for oyster growers because it is important to know the accurate name of species or subspecies that is been cultivated so that it can be marketed with a correct name and on the other hand, the knowledge of the hybrid potential of taxa of oysters for commercial purposes. The detailed knowledge of the genetics of the wild and cultured oysters is fundamental for the adaptation of the organisms to the oyster farm environment because these mollusks have to grow in response to the controlled conditions of the water and their genetic capacities. The participation of the genetics here consists of achieving the modification of the genetic pool of a stock in order to obtain a greater production or better quality of the product through the application of modern genetics procedures. Specific programs should be implemented in order to: a) select the 'best farm animal' and b) maintain the genetic diversity and genetic composition of the wild stock. These are the primary objectives in the process of use management of commercial oysters for their controlled exploitation.

1.6 GENETIC IMPROVEMENT

The feasibility of improving the oyster production by means of its culture and at the same time to assure the conservation of this resource with the participation of the genetics depends on the degree of knowledge on the fundamental aspects that characterize genetic resources of the species and their populations. Emphasis should be done to the knowledge of their basic cytogenetic characters as the background for strategies of genetic manipulation and for the modulation of the ploidies. The

cytogenetic research in oysters has advanced mainly in the determination of the chromosome numbers in the studied species. There are few detailed studies on chromosome bands in spite of the actual existence of good procedures for the obtainment of mitotic fields (Stiles *et al*, 1983; Rodríguez-Romero *et al.,* 1991). The current knowledge on the chromosome numbers in bivalves indicates a range from 2n=14 in *Anomia chinensis* (Anomiidae) up to 48 in *Corbicula leana* (Corbiculidae). In Ostreidae, the characteristic diploid is 2n=20 (Nakamura, 1985). Nevertheless, in most of the works until now published a lack of information on the detailed morphology of chromosome segments is observed. In *C. virginica*, Longwell *et al.* (1967) and Menzel (1968) found biarmed chromosomes and Rodriguez-Romero *et al.* (1978) reported a diploid number (2n)=20, with a 60% of metacentrics and 40% submetacentrics; Rodríguez-Romero *et al.* (1979a,b) accomplished studies on the karyotypes of *C. rhizophorae* of the Atlantic and *C. corteziensis* of the Pacific in those which identified a fundamental number of 40. Studies not yet published on *C. iridescens* of the coast of Oaxaca, indicate the presence of 20 biarmed chromosomes. This information is useful in oyster culture because the potential feasibility of identifying the adaptive changes at chromosome level in the oyster populations and as reference information for the management of gene banks.

In spite of the fact that cytogenetics has demonstrated widely its usefulness in fish and mollusk culture, it has not yet been applied in species of oysters of Mexico. The molecular techniques used in the studies of population genetics provide a powerful tool for the study of the genetic variability and on the geographical distribution of the populations, their hybridization zones and the exchange of genes among them. With the electrophoresis techniques for the analysis of the enzymes as well as by the use of genetic DNA markers the genetic variation in cultivated and wild populations of commercial oysters can be studied to predict the trend of the genetic variation in populations exploited by cultivation. Additionally, this methodology has proven its potential to determine the influence of oceanic currents in the delimiting of the geographical ranges of some aquatic species and is anticipated that it will be of usefulness for the behavioral study of the dynamics of the populations of larvae and adult above all in the edges of the geographic regions that are limited by environmental factors generated by the influence of these marine currents. This circumstance is interesting for example for the confluence zone of the California current with that of Pacific tropical eastern in the Mexican Pacific that defines the Transition Zone of the Tropical Eastern Pacific (Gallegos *et al.,* 1988). The

knowledge of the genetic variability of the wild populations of oysters in the different species of the Mexican coasts will be valuable for novelty strategies of development, as well as in special circumstances as the oyster culture in residual waters that is outlined as a new form of the oysters cultivation, the same has happened with the cultivation of other aquatic species and that each day is but used with success in various parts of the world (Edwards, 1996a, 1996b).

1.7 CONCLUSION

Evidences indicate that the Mexican commercial oysters species of the coasts of the Atlantic and of the Pacific share a common ancestry and that the interspecific differentiation in these organisms has been influenced by the environmental conditions where the populations have been developed. The adaptive processes and the isolation promoted by the continental displacement of the Central American bridge contributed to the slow independent evolution of the populations of oysters isolated since the plio-pleistocene. It is believed that the dynamics of the speciation and the relationships in these oysters could be visualized through multidisciplinary approaches included the genetics and the molecular biology. Due to their abundance and commercialization potential the main species in Mexico are, in the Atlantic, *Crassostrea virginica* Gmelin and *C. rhizophorae* Guilding and in the Pacific, C *corteziensis* Hertlein. For the studies up to now carry out, an incipient speciation is suggested in these species that do not still impede the interspecific genetic flow. The essays of interspecific hybridization and the success in the viability of the hybrids among these three species demonstrate this close parental relationship and an interesting potential for the biotechnological manipulation of their genomes.

The oysters production in live weight including the production by cultivation, represents a 2.74% of the yearly fisheries production; equivalent to the 1.05% of the total value of the nation value of annual fisheries products. In Mexico, the success of the oyster culture in the near future is supported by four species of *Crassostrea*. It is accurate to take into account fundamental aspects that facilitate their operation as an industry with a quality control and a predictable productivity under the following conditions:

1. The species to be cultivated should be scientifically identified taking into account different stages of their biological cycle. This is important for species of the Gulf of Mexico as *C. virginica* and *C.*

rhizophorae because they compete by the same habitat in the coasts of Campeche and Tabasco.

2. It is desirable that the oyster farm includes its own production of seed in the laboratory to improve the quality of the species. It is convenient to locate wild oyster banks with genetically healthy organisms of the species in use to refresh periodically the genetic variability of the stock used for culture. For *C. virginica*, the work of De la Rosa and Rodriguez-Romero (1988) has indicated that due to the genetic variability, the Laguna Madre population is the best option for the regeneration of the genetic pool of other populations along the Mexican coast of the Gulf of Mexico.
3. It is necessary a good knowledge of the biology of the species to be cultured. This information is obtainable from the national research centers of the country in which are accomplished studies on biological oceanography and marine biology. It will be necessary to establish the mechanisms for a good communication between these entities and the oyster farms.
4. The monitoring of the conservation of the genetic quality in the stocks populations on exploitation in benefit of the product quality and the confidence in the forecast of the production. This is an aspect that generally is omitted in the development of the aquaculture projects in Mexico. It is quite convenient to have a specialized reference laboratory that is entrusted with the respective analysis, technical work and the scientific research on the genetic improvement of the species in use.
5. The foundation of aquaculture enterprises based on the stability and quality of the product should consider the participation of the regional human communities in an entrepreneurial development in benefit of their income and of their standard of living. The expected benefits include a better marketing because the oysters cultivation in regions of Mexico that are characterized by a tropical climate are found yet in an initial stage of development and they can reach high productivity levels. At local and regional level this activity can benefit the rural human communities due to the short-term flow of the capital that is generated. It is desirable that the Mexican government and the private initiative support in a decisive form research projects to consolidate the oyster culture as a modern with the best technology installed and the creation of work and income sources for those people which economic condition is critical.

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2

Autonomous Vehicles in Fisheries and Aquaculture

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2.1 INTRODUCTION AND BACKGROUND

The application of modern electronics in aquaculture and fisheries has been growing over several decades and traces back some time (Lee, 2000). The modernization of the world following the industrial revolution has allowed many new opportunities in mechanization and automation, and also contributed to increases in quality of life, length of life, and consequently, to a dramatic increase in world population. In the early 21st century, we are at a crossroads where that population and those techniques have reduced productivity in many ecosystems, again threatening that very population, and also dramatically impacting many other species. This point in history is a moment to question whether to abandon these technologies, to continue their development in a business as usual mode, or to find ways to bend these technologies toward a more environmentally friendly and sustainable future. One such area is automation in environmental areas, including fisheries and aquaculture.

While automated systems have grown to become relatively common in a variety of aquacultural and fisheries areas (Losordo *et al*., 1985; Lee, 2000), the use of autonomous vehicles in aquacultural applications is just beginning (Hall and Price, 2003a-d), and their use in fisheries is likewise in its infancy. The word autonomous is derived from auto, meaning self, and refers to self-guidance. There are certainly a variety of levels of such guidance, and a variety of vehicles, whether boats, airplanes, land based vehicles, submarine or combination vehicles (e.g. water-landing

airplanes, amphibious vehicles), and the potential for even more unique vehicles (outer space vehicles, ski-planes, etc.), many of which have potential application to aquacultural or fisheries operations.

To date, the use of such vehicles in aquaculture appears fairly limited. Hall and Price (2003a,c) used a series of self guided boats to scare predatory birds from aquaculture ponds. Price *et al*. (2005) used GPS guidance to create artificially defined areas so that sensors for physical obstacles were not necessary. Nadimpalli *et al*. (2006) focused on image processing applications in such vehicles, while Hall *et al*. (2007) recognized that measurement of water quality via autonomous boats could also be useful to aquaculture or fisheries applications. Price *et al*. (2003b) used semiautonomous airplanes to capture photographic information to get feedback for agricultural and potentially aquacultural or fisheries (e.g. pond color linked to algae blooms, etc.) applications. Additional developments have been the use of such vehicles to monitor and potentially provide feedback based on water quality, and use of these vehicles in agricultural, aquacultural as well as environmental applications. In addition, a series of underwater autonomous vehicles have been developed (Asakawa *et al*., 1997; Kojima *et al*., 1999; Asai *et al*., 2000), but primarily for use in ship-related applications. However, the potential here is to use similar vehicles for aquaculture or fisheries related operations.

Generally, although autonomous vehicles on land have been developed more extensively with recent examples in the DARPA Grand Challenge (Seetharaman *et al*., 2006), there has been limited development of water based vehicles, and very little work on aquaculturally focused or fisheries focused autonomous vehicles to date. Land based agricultural vehicles have the advantage of a non-moving medium, and minimal visual distortion in air as opposed to water. However, there are challenges with all kinds of moving and especially water based vehicles.

A variety of agriculturally based autonomous vehicles and systems have been developed (Ellis, 1995; Edan, 1995; Kim *et al*., 2000; Noguchi *et al*., 1999; Steward and Tian, 1999; Toda *et al*., 1993; Plaia, 1987). Edan (1995) was one of the first to specifically address design issues in autonomous agricultural robots. Lindgren *et al*. (2002) recognized challenges of power, traction and guidance in land based vehicles and proposed some specific solutions to parts of the problem. Neilsen *et al*. (2002) focused on the potential of such land based autonomous vehicles to be part of precision agriculture systems, and thus hinted at environmentally friendly, low energy autonomous vehicles and their

potential, for example, in deterring weed species without the use of large amounts of costly or dangerous chemicals (Lindgren *et al*., 2002). Wang *et al*. (2004) used neural networks to provide software inspired solutions to guidance challenges on land.

Noguchi *et al*. (1999) focused on design of potentially autonomous agricultural robots, while Tian and Slaughter (1998) recognized the need for specialized guidance software in mobile automated systems. More recently Tian *et al*. (2007) have presented work showing the design process for an agricultural automated vehicle for weed detection and reduction. Nistala considered the use of low level GPS systems to perform ATV (all-terrain vehicle) guidance and site specific sampling in farm fields. This vehicle used a standard WAAS GPS, throttle, steering, and braking actuators, and a special computer system (PDA talking to a single chip controller through a serial connection) to transverse from sampling point to sampling point in a field.

Others who have focused on guidance in aquatic environments include Vincze *et al*. (2003) who worked on a system to guide a robot into a ship, which recognized that water environments present challenges of moving frames of references which land based applications generally do not. Laykin *et al*. (2004) focused on software for recognizing and potentially guiding automated systems, recognizing that novel classification systems (e.g. neural nets, fuzzy classifiers) could provide assistance in this area. Dudek *et al*. (2007) present some of the issues in designing an underwater autonomous vehicle, including a moving frame of reference, various methods for sensing location and environmental parameters, challenges with vision systems (Horgan and Toal, 2006; Broggi *et al*., 2000) in water (e.g. interference, distortion, movement) and methods to deal with them. Prahacs *et al*. (2005) developed an amphibious robot that uses underwater legs rather than traditional control surfaces, allowing it to walk on land or in water. This group also recognized the challenges of moving media such as liquids (or gases), and compensated via inertial reference systems and unique guidance techniques. However, most of these applications do not specifically address the biologically based needs of aquaculture, fisheries and other environmental applications (Hall *et al*., 2007).

2.2. METHODS

Initially, the purposes of certain boats (Price *et al*., 2001; Hall and Price, 2003a,c) were to reduce predatory bird incursions onto aquaculture

ponds. Other possible objectives such as water quality management (Hall *et al*., 2005; Hall *et al*., 2006) have also influenced the design of such devices. However, fundamentally, each autonomous vehicle needs a power source, a mode of movement (e.g. motor and paddlewheel, propeller or legs), a method of navigation, and potentially passive or active sensors for interaction with the environment, for information gathering or other purposes (Figure 2.1). Additionally, some method of processing information (e.g. microchip or similar device) and information storage is necessary. Hall *et al*. (2007) describe a vehicle that captures spatial data by GPS, interacts in a semiautonomous way, via a series of sensors and a radio control override to return the boat to a desired location or guide it to a particular location if needed (Figure 2.1).

Figure 2.1: One version of a series of autonomous boats developed in the Department of Biological and Agricultural Engineering of the LSU AgCenter, here being tested at the LSU AgCenter Aquaculture Research Station in Baton Rouge, Louisiana, USA. This one was large (3x4m, 500kg), and intended to reduce bird incursions on large (e.g. 10-1000 ha) aquaculture ponds and/or drinking water reservoirs. The basic organization still included solar power (not shown); batteries; paddlewheels; microcontrollers and GPS guidance.

Interestingly, some of the multiboat experiments with basic interactions between boats are similar to how humans and other animals interact to do collective projects. Other authors have focused on navigation techniques (Broggi *et al*., 2000) and sensor improvement (Hallset, 1992) or even information management in autonomous vehicles

(Hetzroni *et al*., 2005). Vision systems (Horgan and Toal, 2006; Nadimpalli, 2005) have been the focus of many studies, but continue to provide challenges in moving, refracting environments such as water (Zhang *et al*., 2005) so alternatives, such as acoustic methods (Liu and Milios, 2005) infrared (Mizalkoff, 2003) or inertial techniques (Saez *et al*., 2006; Wang *et al*., 2006; Dudek *et al*., 2007) have been studied.

Possible current and future uses of these boats including the above-mentioned predator reduction (Price *et al*., 2001; Price and Hall, 2002; Hall and Price, 2003a-d; Hall *et al*., 2005), water quality monitoring and management (Hall *et al*., 2006; Hall, 2005; Hall *et al*., 2005), but also include a variety of other environmental management possibilities (Hall, 2005), including in coastal areas (Hall *et al*., 2006). Other purposes may include fish herding or assistance in an automated or semi-automated harvest, monitoring and management of natural fisheries, ferrying supplies to coastal or remote aquaculture locations (especially in rough weather), and monitoring fish stocks across wide areas.

These devices have been or could be used with a variety of species including catfish *Ictalurus punctatus* (Price *et al*., 2001; Hall and Price, 2003a), but also with crustaceans (an automated go-devil for use with crawfish, Price and Hall, unpublished); molluscs (e.g. *Crassostrea virginica* protection from predatory red drum via automated solar powered sonic devices (as has been done by Price and Hall, recently); or potential use of similar devices with rice, algae (recently in the focus as a potential for dramatic production of biomass energy, food, etc.), open ocean ranching of fish or other purposes.

2.3 TYPES OF AUTONOMOUS VEHICLES

A variety of vehicle configurations are possible including floating water based (e.g. boats, Hall and Price, 2003a-d; Mandhani *et al*., 2005; Price *et al*., 2001); underwater vehicles (Kojima *et al*., 1999; Kojima, 2002), amphibious vehicles (Prahacs *et al*., 2005; Dudek *et al*., 2007), and air based autonomous vehicles (Figure 2.2). Airborne autonomous vehicles can be used to capture and analyze photographic information (Figure 2.3) in the visible, UV, IR or other wavelengths to estimate algae growth, water quality, conditions of estuarine areas, or to deliver a (likely very small) dose of some specialized agent. There are also possibilities to use combinations of water and air based vehicles, such as landing on the water to recharge and then taking off again on ski floats; landing to check water quality and taking off (with options for helicopters, guided

or even passive balloons, or airplanes) to continue monitoring from the air; and using air based vehicles to reduce bird predation or perform similar activities on large farm areas (>100 ha).

Figure 2.2: Photograph of semi-autonomous airplane shows self leveling horizon finder (black, center); and GPS (yellow and black/toward rear of plane). Microelectronics and associated control hardware (e.g. servos) are enclosed primarily in center of airplane. A digital camera was mounted in the lower part of the airplane viewing downward (Figure2.3; Photo by R. Price).

Amphibious (Prahacs *et al*., 2005) vehicles could be used, for example, in rice or crawfish culture, where semi-amphibious but not generally autonomous vehicles are currently used. Land based autonomous vehicles could also potentially be useful in traversing near water bodies or in reducing or monitoring runoff or other land-based issues that can affect water quality or aquaculture (Tian *et al*., 2007). The truth is there are many possibilities, and a custom design may be appropriate for each significantly different application. In all cases, objectives may include enhancements to fisheries and aquaculture, via automated techniques, to enhance productivity and sustainability.

2.4 THE LSU AUTONOMOUS AQUACULTURAL VEHICLE FLEET: A CASE STUDY

The Aquacultural Engineering Laboratory of the Department of Biological and Agricultural Engineering (Price and Hall, 2002; Hall *et al*., 2007) at Louisiana State University and the LSU AgCenter has developed a series of autonomous vehicles (Figures 2.1-2.2) which have performed well over a period of roughly six years. The total number of vehicles manufactured or outfitted is now in the double digit range. These include autonomous or semi-autonomous airplanes, boats, submarine and amphibious vehicles or systems. A variety of systems have been deployed and tested, and the successes have opened up more avenues of study and application.

Early vehicles (Price *et al*., 2001) were relatively simple, involving typically a single solar panel, 1-2 rechargeable batteries, a single microchip controller, and physical proximity sensors activated by impact with shore and a concomitant magnetic switch signal to the controller to move the boat out to deeper water and turn back into the pond. These devices were tested for efficacy with reducing bird predation, and found to be effective over periods of 3 days, during approximately 3 months of bird migration activity. Challenges included limitations to power at dawn and dusk; no active visual or other sensors; and no active sound, light or physical means to impact birds.

Later versions of these vehicles incorporated water cannons for active intervention, sensors with variable infrared capabilities, visual cameras and analysis systems (Nadimpalli *et al*., 2006), GPS guidance (Hall and Price, 2003a,b,c); water quality equipment such as dissolved oxygen, temperature, time and location data (Mandhani *et al*., 2005). Additionally, various guidance algorithms, as well as combinations of vehicles have been explored.

Recent data shows that GPS guided vehicles can conserve power, tack into oncoming wind effectively, and be more effective in covering pond areas, thus allowing longer runtime and more effective coverage and reduction of bird predation. Similar capabilities could be useful in capturing water quality or other relevant environmental data, or in the use of such devices in feeding, management or harvest. No data has yet shown any negative effect on fish.

Remembering that autonomy is defined as self guided, this usually requires a ‘brain’ or self guidance scheme based upon set or changing objectives, either self-set or using inputs, direct or indirect, to perform desired functions. This, in turn, suggests a closed loop system with inputs

from the environment. The added challenges of autonomous vehicles is the changing location of the vehicle itself, likely changes in the medium, particularly true for aquatic vehicles, requirement for a portable power source, and lack of direct contact with information exchange, either in (to guide the vehicle) or out (to provide feedback regarding water quality or other relevant variables.

The simplest method used in the LSU fleet was to use magnetic proximity sensors and shore feelers to sense undesired obstacles, reverse course, and change direction. This was referred to as a random course trajectory (Price *et al*., 2001; Hall and Price, 2003a). A similar technique can be used to stay within a pond or virtual boundary based on GPS or other physical feedback defining an undesired area. Similarly, the lack of movement when motors are engaged can be interpreted by the microchip (Price *et al*., 2001; Hall and Price, 2003a), a series of BASIC Stamp chips from Parallax, Inc.). A propeller drive could be used, coupled with a servo to drive a rudder, but a pair of paddlewheels allows tighter turns (by turning one paddlewheel forward and one backward), more predictable turning by the processor, and reduces the interference of algae and pond plants (by operating higher than the bottom depth of the boat).

Another related generalized guidance scheme is to avoid obstacles. For example, this could be to avoid banks on ponds, lakes, rivers or coastal areas; or avoid islands, or other obstacles in open water. More advanced guidance could use GPS or other navigation to follow a line, course, or stay within desired areas. Timing can also be incorporated. Guidance of multiple autonomous entities implies either direct communication, or indirect sensing of other entities, and opens up more interesting possibilities and challenges for guidance. Seetharaman *et al*. (2006) were part of a highly visible group exploring autonomous vehicles in rough, open terrain, and found the challenges significant. In open water the challenges could be even greater. Complex, multiple input / multiple output guidance and interaction systems are being developed and will undoubtedly be significant in future designs.

Energy sources for Hall and Price (2003a-d) were solar, but biomass, battery or fossil fuel for limited periods are also plausible possibilities, and again must be considered depending upon the specific application. It is possible that energy can be rationed and even shared when there are multiple vehicles, yet another permutation on the multiple entity schemes (Kumar, 2000; Hall *et al*., 2006). Regardless, the challenges and opportunities are growing and provide engineers with fascinating

openings in the years ahead. A number of other laboratories have developed their own vehicles, often specializing in a particular type or application (e.g. underwater, aerial, agricultural or military applications), and the results of these growing investigations should be interesting in the years to come, with applications in fisheries and beyond.

2.5 RESULTS TO DATE

Autonomous vehicles have been used for a variety of purposes in aquatic situations. Among the challenges are the current speeds and capabilities of electronics. In this area, it is reasonable to expect that challenges, for example in real time navigation (Nadimpalli *et al*., 2006) will be reduced in the near future, allowing more possibilities for real time interaction of such devices. Accuracy of instruments (Nistala, 2006) is also important, but again, is likely to improve over time.

Among recent results of autonomous vehicles, Hall and Price (2003a) reported that, after analyzing video recordings of bird activity on aquaculture ponds with and without vehicles, or with various numbers of vehicles, bird activity was significantly reduced when vehicles were active. Interestingly, when the vehicles sat idle for long periods, bird activity resumed, suggesting that action is an important part of predation reduction.

More recent work has focused on capturing and analyzing images of birds (Nadimpalli *et al*., 2006), which suggests that certain methods of such image analysis can be effective in discriminating birds from other objects in a pond. Incorporation of such techniques in real-time devices will require either more effective image analysis techniques, or faster processors, or both. However, this suggests that near-real time interaction with birds is possible, and might further reduce incursions of predators in aquacultural or fisheries applications. Use of digital images from satellites or, in cloudy regions, from aerial vehicles, have been used to analyze crop, water, environmental or other variables.

Autonomous aerial vehicles, coupled with digital cameras or video equipment, provide relatively low cost alternatives, which may be more effective and capable of faster feedback for many aquacultural applications (Figure 2.3). Engineering design to match solar power, energy storage via batteries and the physical characteristics of the boat are necessary for effective operation. Recent data suggests that guidance algorithms also can improve pond coverage. For example, in moderate winds (5-15 mph), a ‘heading hold’ technique provided roughly 3-5 times

the distance traveled (5-10km vs. 1-3 km/day) than without. Also, solar power and battery matching (to reduce weight), sunlight sensors, and improved energy management schemes were able to conserve enough energy for 1 to 2 hour run times in the morning and dusk, when solar power was low and predatory birds were still operating.

Figure 2.3: Digital photograph of aquaculture ponds taken from autonomous airplane shows different visual characteristics of ponds, which can be linked to water quality and algae growth in ponds for improved management of aquacultural operations (Photo by R. Price).

Other recent results analyzed a variety of different autonomous water based vehicles developed to date. Parameters analyzed included battery voltage, motor size, pontoons, paddlewheel type and size, solar power and guidance methods. Generally, run time, distance and speed were all higher for heading hold guidance methods, and for higher powered (solar and/or battery) vehicles (although heavier boats tend to increase drag, thus reducing speed and distance traveled). For example, similar boats had average speeds of 300-500 meters/hour for random guidance, while speeds increased to 1000-2000 m/h for some advanced guidance algorithms.

A variety of tasks in aquaculture, fisheries, and related aquatic productivity have been enhanced by use of autonomous or semi-autonomous vehicles. These include water quality assessment, real time interaction with biological entities, reduction of predatory birds, and future applications could potentially include harvest or feeding related

tasks. More could be done, and, as prices change, value of fish stocks will be reassessed in light of increased demand and reduced availability, while availability of higher quality electronics is likely to increase, allowing new possibilities. Similarly, as technologies such as recirculating aquaculture (Timmons *et al*., 2005) and open ocean ranching continue to grow, these technologies will be called into play in more and more areas.

2.6 DISCUSSION AND CONCLUSION

Fascinating opportunities exist in many areas of autonomous vehicle development and research. Energy efficiency is becoming more critical in many other applications, but has been a serious design limitation for autonomous vehicles for some time. Guidance systems and sensors are other areas of continued promise. However, it is not just the technical, but also the philosophical areas that need to be considered.

Concerns that autonomous entities could injure humans, or help humans injure each other or other life forms are serious. However, the possibilities for doing good are also remarkable. Among these considerations are food production, environmental and energy related issues, safety, and medical applications. Some possibilities are that these devices could influence our view of human life, either making us more mindful of its value, or downplaying the need for humans or other creatures at all. Engineers and technologists, as well as theologians and philosophers need to consider and discuss these issues as the technological developments continue. Science fiction is now in many cases, being surpassed by actual technologies, and we would do well to invest intellectual energy in these considerations. Perhaps we will begin, as we realize the abilities, but also the limitations these technologies have, to recognize how precious and remarkable are the wide sets of abilities humans and other animals have to adapt, think and act autonomously.

Autonomous vehicles in aquaculture and fisheries are still in their infancy, but based on results to date, they have the potential for great benefit. There is also a caveat, that self guided vehicles could also have undesired side effects, and designers should also consider this as the field develops. It is likely that such devices will increase in their usefulness and breadth of use in coming decades, and also likely that most of these developments will have beneficial effects on fisheries and aquaculture if wise management and design are considered.

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3

Bioengineered Reefs to Enhance Natural Fisheries and Culture Eastern Oyster (*Crassostrea virginica*) in the Gulf of Mexico

STEVEN G. HALL, ROBERT R. BEINE, TYLER ORTEGO, MATT CAMPBELL, MICHAEL TURLEY

3.1 INTRODUCTION

Aquaculture and development of coastal areas, along with rising sea levels and, in some areas, subsidence of land, have contributed to both the value and the degradation of coastal areas (Penland *et al.*, 2005; Rodriguez *et al.*, 2001). In areas prone to erosion, various impediments to erosion have been erected, often of rock or locally available material. However, these are both costly and tend, in areas of unconsolidated muds, to sink into the local muds, eventually rendering them useless. An alternative concept is the OysterBreak™ (Campbell, 2004; Campbell *et al.*, 2006).

The idea of engineering reefs to take advantage of local flora and fauna, indeed, to enhance their growth, while simultaneously reducing material needs and costs of implementation, are key to the OysterBreak™ concept. Significant work has been done (Campbell, 2004; Ortego, 2006; Hall *et al.*, 2006) and initial emplacements have proven remarkably successful, but design optimization, cost reduction, and long term sustainability need further work.

3.2 METHODS

In order to enhance fisheries and aquaculture techniques, productivity and sustainability of coastal systems, composite concrete structures with special additives, including agricultural byproducts which may enhance oyster settlement (Anderson, 1995) have been produced and emplaced. Part of the technique is understanding oyster biology (Menzel, 1951; Castagna *et al.*, 1996; Newell and Langdon, 1996; Kennedy, 1996; Landgon and Newell, 1996). This technique allows focus on engineering design with the biology (Hall and Lima, 2001; Hall *et al.*, 2005). Multiple designs have been created, including interlocking ring structures (Figure 3.1); stackable bar structures (Figure 3.2); specialized PVC structures with French tubes and other configurations; and substitute oyster shell, made of various admixtures of concrete, gravel, agricultural admixtures including cotton seed, and other additives to enhance hardening and color. The intention is to create a surface and appropriate interstices to enhance bacterial growth and spat set and to create refuge for early oyster spat survival. Further information on specific admixtures is included in Campbell *et al.* (2006), the patent for the OysterBreak™ concept.

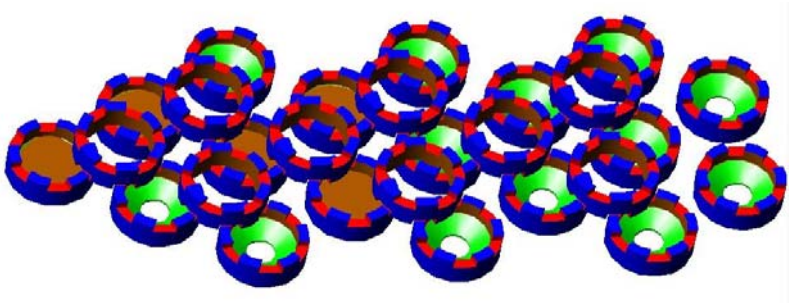

Figure 3.1: Engineering design of interlocking rings have been developed and deployed in coastal and estuarine areas as one method to enhance oyster growth in these areas.

For enhancement of oyster fisheries, there are multiple considerations. These structures themselves, with their unique off-bottom configuration and enhanced surface area, are valuable for growth of oysters, and the spat that these may produce after 1-5 years could contribute significantly to oyster spat availability in and near the area of emplacement, thus jump starting oyster growth in an area decimated by storms, sedimentation, human or disease impacts (Hall *et al.*, 2005).

However, it is also possible to design these structures with removable parts (Figure 3.2) which could enhance harvest after 1-3 years, either as seed for transport to new sites, or for food purposes. In this case, ease of movement and appropriate spacing to allow oysters of expected sizes (e.g. 8-20 cm length) to be removed easily enhance harvest ability. Structural strength to allow the components of the structure to be removed is also essential and is a key design parameter.

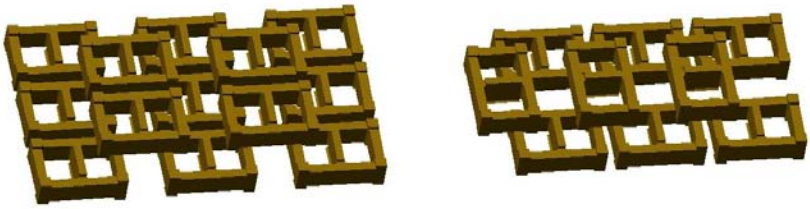

Figure 3.2: Bars or 'logs' of agriculturally enhanced concrete mixes may lend themselves more easily to custom methods of harvesting, but may also tend to have less interlocking initially, reducing their initial stability, but allowing for easy installation.

Additionally, emplacement of these devices in appropriate areas within an estuarine ecosystem is essential. For example, salinity, suspended solids, water flow and other variables appropriate to oyster health must be considered (Wolanski and Spagnol, 2000). On the other hand, in places like Louisiana, where artificial spillways have been and are being built, the sudden changes in salinity may alter the traditional locations appropriate for oysters. These devices can be emplaced at locations that become appropriate (e.g. further upstream or downstream where salinity levels and other variables become conducive to oyster growth) to jump start a new oyster fishery in that area.

Finally, these structures, while proposed to function as submerged wave breaks, could also be partially emerged, providing additional habitat, such as for plants like *Spartina alterniflora* or mangroves (e.g. *Avicenna* sp.) to provide additional protection and visibility if desired for the oysters (Wolanski *et al.*, 2006; Wolanski, 2007). This pairing has the additional potential benefit of enhancing nursery habitat for other juvenile fish and providing additional wave energy reduction for storm protection and erosion reduction in areas near or inland from the OysterBreaks™. Campbell *et al.* (2008, in press), showed that such devices provide significant wave energy reduction, as well as the habitat discussed previously. Additionally, the oyster growth enhances wave

energy dissipation and reduces wave reflection, thus avoiding issues of concomitant erosion often seen with hard protective structures.

3.3 RESULTS

Clearly, these multipurpose, environmentally friendly devices have great potential. To date, studies on strength (Ortego, 2006; Kett, 2000; Mannan and Ganapathy, 2002) suggest that this material is strong enough for desired objectives, and this data has been used in recent design improvements. Growth rates have been observed (Hall *et al.*, 2006) in the range of 5-10 cm per year (Figure 3.3, 3.4), providing the potential for harvest in 2-3 years, and also a potentially sustainable production for food purposes as well.

Figure 3.3: A photograph of both interlocking rings and bars with encased oysters reveals the desired texture: capable of providing refuge for young oysters and good reduction of wave energy, while locking or stacking together.

Figure 3.4: Growth observed on biologically enhanced engineered bars after approximately 4 months of growth suggests annual growth rates in the range of 5-10 cm, with 2-4 layers of interlocking oysters cementing components together.

The coastal protection potential (Ahrens, 1987; Campbell, 2004; Hall *et al.*, 2006; Campbell, 2008, in press) benefits are, from the point of view of fisheries and aquaculture, mostly additional to direct oyster productivity, but in areas where high rates of erosion are occurring, the protection of the estuarine environment by coastward versions of these devices may be as significant as the direct productivity of oysters on the structures.

Timing of emplacement is another critical variable. Because natural spat is highly available for 2-3 periods during the warmer months, it is important to emplace just before or during these periods to allow oysters to colonize quickly. Otherwise, other encrusting organisms such as barnacles or tubeworms, or layers of biofilm or algae may dominate the initial colonization and potentially slow the growth of oysters at a later date.

While work is ongoing, emplacements and experiments to date suggest that the OysterBreak™ is potentially a low cost, environmentally friendly, multipurpose technique to produce oysters for food and to simultaneously enhance natural fisheries in coastal estuarine ecosystems (Figure 3.5-3.6). Emplacement techniques are currently an area of further development. While small emplacements (Figure 3.6) can be done by hand or with small equipment, proposed large emplacements (hundreds to thousands of meters) will require engineered techniques for emplacement that will keep the technique safe, environmentally friendly and cost effective.

Figure 3.5: Growth of oysters shows further enhancement of the porous interlocking structure which allows for habitat as well as wave energy reduction.

Figure 3.6: Emplacement in estuarine waters in southern Lousiana shows an intertidal placement (rings are slightly emerged at low tide, and may be submerged during high water).

3.4 DISCUSSION AND CONCLUSION

The potential for enhanced aquaculture and coastal fisheries based on submerged bioengineered oyster dominated wave breaks is significant. Opportunities to improve existing productivity, to quickly restore or rehabilitate areas which have had reduced productivity due to storms, changes in hydrology, over fishing or other impacts fit well with engineered reefs. Concerns include careful emplacement to avoid undesired changes to estuarine ecohydrology (Wolanski, 2007), potential for impediments or danger to recreational boaters, or to shipping, and the potential for introducing undesired species, materials or diseases. All of these must be considered when designing and emplacing such devices. However, the potential benefits are manifold: enhanced oyster growth, rates, harvest ability, and potentially quality; enhanced coastal fisheries; reduced wave energy, and thus the possibility of preserving valuable coastal wetlands; and improved storm protection for nearby coastal communities. Finally, the cost for these devices should be significantly less than traditional hard coastal protection structures, for at least two reasons: they require much less material (Hall *et al.*, 2006) and due to their lower mass, emplacement should be easier.

While relatively early in their development, bioengineered oyster dominated reefs, termed OysterBreak™, can provide a workable and environmentally friendly method to enhance oyster productivity and coastal fisheries in appropriate areas such as the Louisiana Gulf coast. Further development should optimize the balance between oyster

productivity, enhancements to local fisheries, reduction of erosion, and storm protection, based on the unique requirements of each locality. Developing a technique for this type of custom design is another area of future work, resulting in a more productive and sustainable coastal environment.

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4

Engineering Natural and Artificial Reefs in Oyster and Coral Dominated Environments

STEVEN G. HALL

4.1 INTRODUCTION

Aquaculture and development of coastal areas, along with rising sea levels and, in some areas, subsidence of land, have contributed to both the value and the degradation of coastal areas. In areas prone to erosion, various impediments to erosion have been erected, often of rock or locally available material.

The idea of engineering reefs to take advantage of local flora and fauna, indeed, to enhance their growth, while simultaneously reducing material needs and costs of implementation, are key to the oyster break concept. Previous work (Campbell, 2004; Campbell and Hall, 2003; Ortego, 2006; Hall *et al*., 2006) has focused on oyster dominated artificial reefs as opposed to hard structures (Ahrens, 1987), and with good preliminary success. Other potentially related investigations have included work on artificial coral reef (Dinsdale, 2003; ReefBalls™, 2007; Sherman *et al*., 2002) but the idea of combining artificial submerged or emerged breakwaters with native plant species is a newer engineering challenge. Submerged plants such as eelgrass, or emerged coastal plants such as *Spartina alterniflora* or various mangroves, capable of living in salt water, due to their unique salt removing processes and root systems, provide both important ecological and hydrological functions. As such, they have the potential to be incorporated into both natural and artificial restoration or rehabilitation projects. The roots slow erosion and provide

habitat for a variety of fish, molluscs, crustaceans, sponges, bacteria, algae and other forms of life. Due to the size of the interstices between roots, a variety of small organisms can survive here, and these are known to provide nursery grounds for a variety of important food species. Finally, in a mud-dominated ecosystem, mangroves and other plants may also reduce or reverse erosion, and simultaneously filter and clean the water.

A parallel concept can be explored in coral dominated ecosystems, keeping in mind that coral, as reef building organisms, have similar potential for stabilizing marine ecosystems, but, with their symbiotic algae, require sunlight. As such, they are found in clearer waters. This fact also influences methods to engineer with them, as reducing excess flows of nutrients and especially of muds is necessary to enhance their growth. An example of a structure which could be used with corals, oysters, or potentially combined with appropriate marine or coastal plants is shown in Figure 4.1. Finally, recent findings (Langdon *et al.*, 2007; Hoegh-Guldberg, 1999) and the general recognition of atmospheric changes in carbon dioxide, with the likely acidification of the ocean, must be considered in any reef building exercise. Finally, combinations of engineered reef components should recognize the advantages of each, allowing roots to grow and filter feeders to build where there is ample sediment, and possibly reducing these flows to areas where coral may thrive. From an engineering perspective, reducing economic and ecological costs while maximizing ecological services and enhancing biological diversity are not necessarily mutually exclusive design objectives.

Figure 4.1: The engineered reef design shown requires 80% less material than a solid reef; provides habitat and surface area for reef builders such as oyster or coral to grow, and is much lighter than solid materials, reducing cost and enabling quick emplacement.

4.2 METHODS

In biological or ecological engineering, one of the first principles is to understand the biological system, and thus to design with the biology, making systems more effective and sustainable (Hall and Lima, 2001; Hall 2002). Understanding first the biology of desired sessile organisms, and then appropriate pairings with plant species is thus appropriate. Oysters, as filter feeders, tend to do best where sediment flows and muddy waters provide appropriate food (Castagna *et al*., 1996; Campbell, 2004; Hall *et al*., 2006; Kennedy, 1996; Langdon and Newell, 1996), while corals tend to do better where waters are clearer. As to considerations of plants, mangrove biology and ecology is essential to improving the engineering success of mangrove dominated or mangrove-inclusive engineered restoration projects. Lewis (1990) gives a good overview of mangrove biology, including descriptions of the salinity levels (20-100 typically, depending upon species and other environmental variables), temperatures (tropical, subtropical) and nutrient requirements of mangroves, as well as more complex considerations for optimizing fish habitat, for example. This and other studies (Duke, 2006; Spalding, 1997; Lewis and Gilmore, 2007; Mumby *et al*., 2004) and considerations of the engineering implications (locations with appropriate salinity, water quality, temperature; growth rates; strength and other physical characteristics of growing and mature mangroves) of these unique plants are essential to successful use of these as part of ecological or biological engineering solutions to coastal challenges worldwide.

One concern that is now coming to the forefront are global changes in atmospheric or oceanic parameters. One example is temperature, which may affect many life processes by enhancing enzyme activity, or encouraging diseases. Another example is carbon dioxide which, in ocean water, may reduce pH, and hence impacting ocean processes, including potentially reef building. Thus, while we propose using reefs to stabilize carbon levels, as well as provide other services, those same reefs may be impacted both in their growth phases (e.g. the level of calcium carbonate fixed from ocean water) as well as in their storage phases. It seems intuitive that additional carbon may enhance many forms of carbon fixation, while the counterintuitive possibility exists that some species may be negatively impacted by changes in pH, temperature or other related parameters, possibly leading to losses in reef mass, instead of gains. Hence, engineers may be consulted with greater urgency regarding methods to reduce or reverse these trends. Well designed engineered

reefs, whether in coral, oyster or other ecosystems, may become even more essential than at the current time.

4.3 CORRELATING ENGINEERING DESIGN OBJECTIVES WITH SYSTEM OPTIMIZATION

Another focal point for good engineering design is a clear definition of the desired objectives or outcomes (Hall and Lima, 2001). In this case, the purpose of an engineered system may include a variety of purposes, from food production to wave reduction to water quality improvement or fisheries enhancement. Based upon a specific design objective, the form of a given artificial reef system or mangrove dominated system may differ significantly.

If purposes are primarily wave reduction or erosion reversal, then high densities and relatively quick growth rates could be considered positive. However, structural strength is often inversely correlated with growth rates in biological systems. As a result, the expected structural strength and related physical properties of the combination system (e.g. artificial structure plus added mangrove, oyster or other biomass) must be considered. Limited models are currently available for estimation of such strength (Hall and Campbell, 2003; Cambpell *et al*., 2008), and future work should include consideration of structural strength of composite biological structures (Washa, 1998; Kett, 2000; Mannan and Ganapathy, 2002; Ortego, 2006) over time.

Another possible objective is food production (e.g. from oysters). In this case, both growth rate and form or availability of the food animal is important. One may also consider not only instantaneous productivity, but consistency and sustainability (robustness of the overall system and population) over time (Campbell *et al*., 2008, in press).

Finally, at different locations in the world, objectives may be quite different. For example, ecotourism in areas of coral growth or mangrove forests may mean that the encouraged growth is not expected to be heavily harvested, but that a more pristine area with diverse wildlife and a healthy habitat may be desired (Figure 4.2). In these cases, by contrast with areas where oyster growth may be dominant, remarkably clear water (or only stained by tannins as in the case of mangroves) may be found, and may well be desirable for viewing fish or other organisms of interest. Additionally, in each of these cases, the hydrology, as well as the ecology of the area is important.

Figure 4.2: Mangroves, with their ability to survive high salinities, and with extensive root systems, can provide habitat while reducing erosion. The Rhizophora or red mangrove shown has prop roots which can be several meters long.

In coastal areas, tidal fluxes move nutrients, provide oxygen, alter microhabitats on a regular basis, and can dramatically affect such parameters as salinity. Additionally, input from rainfall, riverine inputs, as well as normal waves and storm surges (and their frequencies) all play a part in several critical parameters for sessile animals or plants. Solids (e.g. TSS, TDS) are obviously moved more vigorously during periods of greater water movement, but this has a potential positive impact (up to a point) on oysters, while it may have a more negative impact on coral (Ayling and Ayling, 1999). Extreme events tend to move sediment dramatically, potentially burying bottom dwelling oysters, corals and possibly altering the hydrology around mangroves. While it may be impossible to predict extreme events far into the future, using historical data, Monte Carlo simulations or other models to account for likely changes is appropriate engineering design. These models should also include water quality parameters and likely changes in these such as salinity, temperature, dissolved oxygen (DO), nutrients and others.

Another aspect of good engineering design is recognition of the ecosystem type - the native plants, animals, sessile reef building organisms in the area. Expected speed of growth, which is itself influenced by a combination of temperature, nutrients, dissolved oxygen or other limiting factors, and the organisms themselves, is a critical variable. For example, in design of the oyster break (Hall *et al*., 2006), a part of the engineering model developed by Campbell (2004)

incorporated expected growth rates of oysters and the resulting impact on wave reduction, mass, and sedimentation. As a practical result, this may impact the spacing of components in an artificial reef, and could influence sustainable harvesting from natural reefs as well.

With changing water levels, either due to sea level change (e.g. rise) or subsidence of unconsolidated muds or other geological structures, the growth rates of natural or artificial reefs can allow organisms to continue to survive at optimal depths, or in some cases, may provide for the slow sinkage of reefs. Ancient reefs are found in many parts of the world at depths which make them unproductive. Reef design should consider methods to allow growth at optimal levels, for example, starting with components of the reef in an emerged configuration, and allowing for submergence over a period of years, with concomitant growth on the sections at optimal height at a given time allowing the reef to remain healthy for many decades.

Mechanical strength requirements are an apparently simple, yet complex consideration. For example, encrusting organisms do at least two things to a reef structure: they add strength by cementing themselves in various ways to the structure and to each other. However they also add mass which increases stresses in structural members. In addition, the largest outside force - that of wave energy - is increased, possibly dramatically, as organisms grow and provide more surface area for waves to impact. Engineering design should consider the expected failure modes, and resulting impacts on ecology, wave energy, sedimentation, and future growth of the reefs. Allowing failure to occur under some circumstances may well be a design feature, and a balance between increased strength and initial cost is an essential engineering design feature.

Growth of sessile organisms (e.g. corals, oysters) will also affect mass and hence sinkage rate in unconsolidated muds. In addition, growth rate, and hence sinkage rate, may change over time, providing for nonlinear phenomena and variable rates of movement of the resulting structures. Hall (2002) recognized the engineering challenges of nonlinearities in biological systems and suggested possible solutions, which could be used in these cases. On the other hand, increased mass and increased depth in supporting muds generally correlate with reduced lateral movement, meaning that reefs gain increased lateral stability over time, another engineering parameter of great interest in areas of movement such as coastal barrier islands.

Another unique consideration is the recognition of predatory organisms (e.g. oyster drills or Black Drum fish predation on oysters in

the Gulf of Mexico) which can impact net growth rates by physically removing or killing desired sessile organisms. Engineered reefs may have exclusion devices such as nets, limited access to crawling organisms via posts and wires, as in typical longline systems, or other methods to reduce these impacts.

Introduced species (e.g. zebra mussels) which may appear to be a nuisance, could be used to good effect in certain places by providing well engineered structures for desirable growth. One must also allow for the increased spat production from enhanced reefs, which may be seen as positive (e.g. increased oyster spat in desirable oyster grounds) or negative (increased zebra mussel reproduction in areas where they are a nuisance). Nevertheless, recognition of these factors is necessary to good engineering design.

Another way that engineers can take an apparent negative and potentially use this for positive ecological results may be the use of certain species (e.g. some of the more sensitive corals or certain reef species) as indicators for pollution. Indicator organisms are often used by ecologists, but engineers could also design reefs to make the health of such organisms quite apparent, essentially designing a reef-sized ecosystem health indicator. In all these ways, opportunities for good engineering design in reef systems are available.

4.4 RESULTS TO DATE

Engineering design requires appropriate design objectives and knowledge of design parameters (Hall, 2002). A variety of artificial reefs have been emplaced with some success at cultivating desired species (Hall *et al*., 2006; Campbell *et al*., 2006). However, actual engineering design of reefs is as yet a limited process. Reef balls (reefballs.org) are a notable, if limited example of design that provides appropriate substrate for the desired coral to affix to. These are also designed with appropriate spacing to encourage desired reef species of fish to congregate and use the spaces. However, they are limited in size (e.g. individual balls up to approximately 1 meter), and are still relatively heavy and thus inappropriate for areas without reasonably hard bottoms.

An alternative development is the oyster break technology (Figure 4.3; www.oratechnologies.com), which also provides appropriate substrate, and, due to its agricultural byproduct inclusions, may even enhance settlement of oysters and other desired organisms (Anderson, 1995; Hall *et al*., 2005; Ortego, 2006; Hall *et al*., 2006). However, due to

the various geometries which have been explored with the oyster break technique, larger structures can be built with interlocking parts, allowing more stable assemblages, which are critically important in areas of storm and wave imposition. Also, the oyster breaks can be built with variable density materials, and the overall density is an explicit design parameter, allowing them to be more appropriately and successfully emplaced in areas of movable muds or soft sediments.

Figure 4.3: Oysters can be grown on engineered materials as shown. These components should be assembled in a way designed to optimize the results for the desired objectives.

These techniques may also be enhanced by use of plant material. Plants which thrive in estuarine waters may include grasses (e.g. *Spartina alterniflora*) and trees (e.g. mangroves such as *Avicennia germinans*). By combining these species with artificial reef structures, mixed assemblages of plants and animals can be nearly instantaneously emplaced, allowing rehabilitation of areas that might not be possible at all, or with this speed by normal settlement patterns. This could be critical in areas with altered hydrology (e.g. riverine estuaries with new spillways, or areas cut by development of roads in coastal mangrove habitats), where natural processes of recovery could take so long that other species that require this habitat (e.g. waterfowl, crustaceans, juvenile fish) would die out in the area. Additionally, the storm protection that is provided by natural reefs and coastal plants is well documented. By restarting these natural processes, other areas, inhabitated by humans or others would be protected much faster, reducing losses of life and homes.

Thus, multipurpose artificial reefs, or focused purpose reefs can be designed with consideration of the complex and interacting engineering parameters including water quality, physical parameters, and biological growth rates, interactions and strengths to provide desired ecological results. In some cases this may be to enhance growth of a harvestable species (e.g. Campbell, 2004; Hall *et al*., 2006), while in other cases, it may be to rehabilitate an area after human or natural impacts have dramatically altered a system (Lewis, 1990; Lorenz *et al.,* 2002; Lewis and Gilmore, 2007). In any case, the recognition that multiple interacting physical and biological parameters must be considered both improves and complicates the engineering design process.

4.5 DISCUSSION AND CONCLUSION

A more in depth consideration of parameters and ecology is necessary for this work to continue to move forward. This means that engineers and biological scientists need to work together. Engineers can provide possibilities to scientists, showing what-ifs that are critical to good rehabilitation of degraded ecosystems, while scientists can focus on needed engineering parameters (e.g. growth rates, physical strength) which may not have been recorded previously due to their limited usefulness from a purely biological point of view. Together, engineers and scientists have many opportunities to restore degraded environments, or to reduce destruction when anthropogenic (e.g. dredging, spillways) or natural (e.g. storm events, sea level changes) changes impact a given ecosystem.

There are also concerns and challenges. To some extent, the fact that changes have already been made in so many places means that the pure preservation approach is inappropriate, and moves the interested scientist or engineer toward improved interactions and active and thoughtful rehabilitation and restoration efforts. Recognizing that additional changes due to anthropogenic (e.g. agricultural runoff; altered atmospheric and marine chemistry) and natural (e.g. long term solar cycles) changes allows earlier and less expensive improvements to maintain or rehabilitate ecosystems to maintain essential ecosystem services while also providing for human needs. Short term improvements include reduced erosion, growth of desirable species for harvest or for their services (e.g. water filtration), enhanced ecosystem functions, and enhanced growth of species that use reefs. Longer term considerations include current bleaching of coral reefs and methods to engineer

improvements there, and considerations of long term changes to ocean level and/or chemistry with the need for appropriate engineering design to minimize detrimental impacts of these changes. In conclusion, improved engineering design of reefs, whether using coral or oysters, is desirable, and may be necessary in many areas to maintain or enhance coastal health.

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5

Application of DNA Fingerprints in Study of Seaweeds

DELIN DUAN

5.1 INTRODUCTION

New molecular advances have be made in a range of biodiversities (Janson and Buckler, 2007), genetic mapping (Malyshev and Kartel, 1997), marker assisted breeding, genome fingerprinting and functional gene study. Rapid progress has been made in algal molecular biology since DNA fingerprint techniques have been applied in seaweed study. Polymerase chain reaction (PCR) is widely used in algal genomic DNA analysis, with one of its main application being in the development of DNA makers for map construction useful in breeding, taxonomy (Manhart and McCourt, 1992), evolution systematic and evolution (Fain, 1988; Olsen, 1990) and gene cloning (Deng *et al.*, 2004). Currently, various molecular markers serve mainly for the application of germplasm identification and genetic analysis and for illustrating algal phylogenies, evolutional and molecular genetic mechanism (Arai *et al*., 1995).

Molecular markers-assistant selection in seaweed breeding and cultivation requires more effective molecular markers for seaweeds. Compared with the isoenzyme (Wang *et al*., 1999; Sosa *et al.*, 1993, 1999) and morphological features, application of DNA marker systems should be prioritized, because many genetic loci can be detected in a segregated population and a linkage map with high density may be established. The content of polymorphoric data is higher than that of morphological character and is neutral to the phenotype. Single locus content appears much higher in dominant character or recessive

character and molecular markers are usually co-dominant, except for the random amplified polymorphic DNA (RAPD) marker. DNA molecular markers appear on histological or cellular levels in the organisms and are detectable in any developmental stage but morphological character can be observed only at certain developmental stages. Therefore, molecular markers can expedite the selection of good characteristics for seaweed cultivars.

Applying molecular markers provides an opportunity to reveal algal DNA sequence polymorphism useful for discriminating genetic variation among individuals and within populations of seaweeds (Alberto *et al.*, 1997; Niwa *et al.*, 2004; Sosa *et al.*, 1996; Zhao *et al.*, 2007a,b). Several PCR marker systems with varying complexity, reliability and information could be generated. RFLP and RAPD have been used extensively to characterize plant genetic resources (Rafalski *et al*., 1991; Nagaoka and Ogihara, 1997), but there is little documentation related to molecular assistant-selection for seaweed breeding. DNA fingerprint analysis for genetic and variation survey of seaweeds will be significant in seaweed germplasm analysis and breeding in future.

5.2 DNA FINGERPRINTING METHODS

5.2.1 Restriction Fragment Length Polymorphism (RFLP) Marker

RFLP marker in plant breeding analysis (Beckmann and Soller, 1983) is one of most popular genomic fingerprinting techniques applied in the study of plant breeding. For DNA fingerprint application, RFLP marking is possibly the earliest method applied in seaweed (Fain, 1988). This method can be used to analyze genome DNA, digestion of DNA by site-specific restriction enzymes and subsequent size separation of resulting fragments by agarose gel electrophoresis. RFLP analysis is informative and reproducible, but it is laborious and requires relatively large quantities of DNA (10 ug). Disadvantages of RFLP include the need for using isotopes and the amount of time consumed.

5.2.2 Random Amplified Polymorphic DNA (RAPD) Marker

RAPD technique (Williams *et al*., 1990; Welsh and McClelland, 1990) offers advantages over RFLP, because it is not necessary to know the genomic information previously, and it is only required a small amount of

DNA (10-25 ng) for experiments. However, RAPD marker is sensitive to slight variations in reaction conditions and reproducibility is a concern (Malyshev and Kartel, 1997). Van Oppen (1996) believed that RAPD signal is strong enough and more useful at larger biogeographic scales than at fine population levels. RAPD markers signals are 4-6 times stronger than RFLP markers and require no isotope but RAPD markers can not detect amplified products with gene type AA and Aa due to single gene locus. Moreover, there is poor consistency and low multiplexing output limits, causing varying results in different labs. Nevertheless, this marker has featured in much work on seaweed genetic variation (Patway and van der Meer, 1994; Dutcher and Kapraun, 1994; Ho *et al.*, 1995; Wang *et al.*, 2004; Wang *et al.*, 2006) and genetic mapping (Haring *et al.*, 1996).

5.2.3 Amplified Fragment Length Polymorphism (AFLP) Marker

AFLP method is an efficient DNA fingerprinting technique (Vos, 1995) with high multiplexing ration and is now widely used for plant genomic fingerprinting. AFLP is based on selective PCR amplification of restriction fragments from a total digestion of genome DNA with *Eco*RI and *Mse*I. It involves three steps: 1/ restriction of the DNA and ligation of oligonucleotide adapters, 2/ selective amplification of sets of restriction fragments, and 3/ gel analysis of amplified fragments. PCR amplification of restriction fragments is achieved by using the adapter and restriction site sequence as target sites for primer annealing. Selective amplification is achieved by the use of primers that extend into the restriction fragments, amplifying only those fragments in which the primer extension match the nucleotides flanking the restriction sites. The main disadvantage of the AFLP method is its complex multiple steps including DNA digestion, ligation and amplification. So far, AFLP marker has been applied in the population genetic study of *Chondrus crispus* Stackh (Donaldson *et al*., 1998), genetic differences between two growth-forms of *Lithophyllum margaritae* (Schaeffer *et al.*, 2002), genetic structure analysis of *Alaria marginata* (Kusumo and Druehl, 2000), *Porphyra* cultivar background analysis (Sun *et al.*, 2005) and genetic map of *Laminaria* (Li e*t al.*, 2007).

5.2.4 Simple Sequence Repeats (SSR) and I nter-Simple Sequence Repeat (ISSR) Marker

ISSR techniques originated from the SSR method and involve SSR primers complementary to microsatellite PCR-amplify regions between microsatellite loci rather than providing information about variation in a particular microsatellite locus (Zietkiweicz *et al*., 1994). ISSR techniques contain several repeated nucleotide sequences (2-6 bp) and make up several simple sequences or minisatellite sequences (GA)n, (AC)n. These repeat sequences are dispersed on the chromosome and result in the polymorphic for each locus. The resultant sequence tagged microsatellite usually identifies a single locus and is multi-allelic because of the high mutation rate of SSRs. Alleles differ in base pairs and can be resolved on agarose gels for detection.

Usually, ISSR technique generates 3-5 times the variation of RAPD (bands/markers) while having greater reproducibility and equal cost (Nagaoka and Ogihara, 1997). ISSR markers have been shown to be more reliable and conform more closely to dominant Mendelian inheritance than RAPD markers. This method is useful for genotype analysis and genome mapping. SSR markers can be used as a probe for detecting repeat sequences in animal or plant genomes.

The disadvantages of SSR markers are that single copy sequences must be found and sequenced on each chromosome locus and restriction enzyme digestion and hybridization should be conducted with designed primer. Morgan (1999) reported that the application of SSR markers proved useful for discriminating seaweeds and thereafter, many studies were documented on *Undaria* lines (Wang *et al.*, 2006), *Porphyra* lines (Sun *et al.*, 2006) and *Laminaria* lines (Wang *et al.*, 2005).

5.2.5 Sequence Characterized Amplified Region (SCAR) Marker

To overcome the stable reliabilities of RAPD analysis and improve DNA marker assistant selection and map-based cloning, some special RAPD loci are usually converted into a sequence characterized amplified region (SCAR). By designing specific primers based on the sequence data and amplifying DNA loci under stringent PCR conditions, SCARs have been applied in the study of several plants (Paran and Michelmore, 1993; Witsenboer *et al*., 1995). SCAR marker has been constructed for *Porphyra* (Sun *et al.*, 2005), *Undaria* (Wang *et al.*, 2007) and *Laminaria* (Wang *et al.*, 2004). SCAR conversion significantly improves the reproducibility

and reliability of PCR assays and allows development of co-dominant PCR-based markers for seaweed selection.

5.2.6 Sequence-Related Amplified Polymorphism (SRAP) and Target Region Amplified Polymorphism (TRAP) Markers

Sequence-related amplified polymorphism (SRAP) and target region amplification polymorphism (TRAP) systems are two newly developed molecular markers in plants (Lin *et al.*, 2004; Hu *et al.*, 2005). SRAP aims for the amplification of open reading frames (ORFs) by using pairs of arbitrary primers, so its amplification products are distributed arbitrarily across the genome (Li and Quiros, 2001). TRAP is a PCR based molecular marker technique developed on the basis of SRAP. Two primers consisting of 17-21 nucleotides (nt) are used in TRAP analysis: a fixed primer is designed from the targeted expressed sequence tag (EST) sequence from the database and an arbitrary primer is an arbitrary sequence with either an AT- or GC-rich core to anneal with an intron or exon, respectively (Hu and Vick, 2003). The forward primers preferentially amplify exonic regions and the reverse primers preferentially amplify intronic regions and regions with promoters. The observed polymorphism originates in the variation of the length of these extrons, introns, promoters and spacers among individuals and among species (Li and Quiros, 2001; Ferriol *et al.*, 2003a). The SRAP marker system has been used to investigate genetic diversity in plant species, including *Brassica* (Li and Quiros, 2001), *Cucurbita* (Ferriol *et al.*, 2003 a,b), buffalograss (Budak *et al.*, 2004), cotton (Lin *et al.*, 2004) etc. TRAP has been used successfully for fingerprinting lettuce cultivars (Hu *et al.*, 2005) and constructing genetic linkage maps in wheat (Liu *et al*., 2005) and sunflower (Chen *et al.*, 2006). Few papers provide documentation on seaweeds *Porphyra* (Qiao *et al.*, 2007a,b), but it is hoped that this method will be applied more widely in seaweed study.

5.2.7 mRNA Differential Display Markers

Apart from cDNA library construction (Collén *et al*., 2007) and functional gene verification from the genomic library (Deng *et al.,* 2004), mRNA differential display is a new way for verifying genes which correlated with the phenotypes or expressed in certain developmental stages. Liang and Pardee (1992) introduced mRNA differential display with genes expressed differently in various cells or under altered conditions. The crucial step of this method is use of a set of oligonucleotide primers, one

anchored to the polyadenylate tail of a subset of mRNAs, the other short and arbitrary in sequence so that it anneals at different position relative to the first primer. Recent improvements have been made for optimizing primer design and reaction cycle and excluding pseudo-positive clones. Lisitsyn *et al*. (1993) devised improvements and made this method more valid. Hong *et al*. (1995) applied the method to tissue-specific mRNAs in *Porphyra perforata*. Jin *et al*. (2004) detected acid-inducible genes from *Porphyra yezoensis*, either Kang *et al*. (2006) isolated pollutant (pine needle ash)-responding genes from *Ulva pertusa*.

5.2.8 Single Nucleotide Polymorphism (SNP) Markers

High SNP frequencies exist in most genomes and theoretically, there are four allelic genes on each SNP site (A, G, T, C). However, SNP only exhibits two allelic genes (A/T, G/C), so maximum heterogeneities should be 50%. Even though its information is much less than that of an ordinary DNA marker system, SNP is more convenient and promising. Zuccarello *et al.* (1999) applied SSCP to plastid variation of *Bostrychia montagne* and proved that SSCP was effective in detecting plastid variation. Batley and Hayes (2003) applied single nucleotide polymorphism genotyping to *Nodularia*, and showed that SNP could be applied to genetic analysis of bulk populations.

5.3 APPLICATION IN SEAWEEDS STUDY

5.3.1 Germplasm Examining

In recent years, much work has been conducted on identifying germplasm by applying RAPD, SSR and ISSR markers (Havey, 1995). Ho *et al.* (1995) compared DNA polymorphorisms for 13 samples of *Sargassum* collected around Malaysia and Singapore and examined individual samples within the genus. Analysis proved that DNA markers can be effective for discriminating cultivars of *Porphyra* (Sun *et al.*, 2005), *Laminaria* (Wang *et al.*, 2005) and *Undaria* (Wang *et al.*, 2006). Usually, RAPD is used to assess the germplasm of commercial seaweed cultivars crucial for the seaweed preservation and discrimination.

5.3.2 Phylogeny and Evolution Study

Much documentation focuses on seaweed phylogenic and evolutionary studies. At present, it is hard to verify species merely according to the classical taxonomy charactistics; molecular data must be combined with

morphological characters for taxonomic and molecular phylogenic analysis. Manhart and McCourt (1992) pointed out that species concepts are based on morphological, biological and phylogenic views. From the evolutional points of views, molecular data (5s rDNA, 18s rDNA, 16s rDNA, and ITS sequences) can provide information for phylogenic study, and many documentations had been addressed on this subject in the past twenty years (Edvardsen *et al.,* 2000; Blomster *et al*., 1998; Hu *et al.*, 2007a,b).

5.3.3 Genetic Variation Analysis

Using available DNA markers, much work has been conducted on genetic variation (Bouza *et al*., 2006; Coyer *et al*., 1997; Engelen *et al*., 2001; Faugeron *et al*., 2004). Dutcher (1994) studied genetic variation in three species of *Porphyra* and indicated 40- 60% similarities among species along the coast of North Atlantic Ocean and Gulf of Mexico. Alberto *et al.* (1997) examined three populations of *Gelidium sesquipedale* with RAPD markers on the southern coast of Portugal, concluding that RAPD is useful for examining genetic diversity of G*elidium*. Coyer (1997) analyzed the genetic variation for *Postelsia palmaeformis*. Zhao *et al.* (2007, 2008) examined *Sargassum thunbergii* and *S. muticum* populations, showing that high genetic structuring among the four *S. muticum* populations along the distant locations was clearly indicated in RAPD and ISSR analyses. Wang (2008) applied ISSR markers to examine genetic variation of *Chondrus* Stackhouse populations from North Atlantic, and suggested that genetic differentiation between nine *C. crispus* populations closely relates to geographic distance on larger distance scale (ca. >1000 km). The ISSR markers application proved to be useful for determining genetic differentiations of *C. crispus* populations including morphologically inseparable haploid and diploid individuals.

5.3.4 Constructing the Genetic Map

Gene mapping is important for the molecular study in genetics and due to the rapid and efficient markers, RAPD method can be applied to gene mapping. Haring *et al.* (1996) studied sexual behavior with markers for the heterothallic *Chlamydomonas eugametos* and indicated that about 80% of markers represented unique DNA sequence, among them, 15 RAPD markers and one locus with RFLP markers were constructed, and this systems could be possibly applied to analysis for sexual behavior. In addition, Li *et al.* (2007) constructed the first genetic mapping of

Laminaria japonica and L. longissima using the 'two-way pseudo-testcross' strategy, and it is expected that one specific genetic map which closely related with some characters of commercial seaweeds could be finished, because it is crucial to the gene fixation from the seaweeds.

5.3.5 Molecular Marker-Assistant Selection (MAS)

Molecular marker-assistant selection is the ultimate goal for the DNA fingerprinting analysis. Molecular marker-assistant selection has been widely applied to breeding plants with specific character (Havey *et al.*, 1995), but there are few reports on identification of seaweed characters correlated with gene locus. Patwary *et al.* (1994) examined *Gelidium vagum* with RAPD markers and identified the presence of male-specific RAPD markers. By collected individuals from 129 x 130 hybrids, molecular marker-assistant selection exhibited a 9.5-130% higher growth rate. It suggests the presence of heterosis, and indicates that MAS can be applied in seaweed breeding.

5.4 CONCLUSION

New molecular technological developments will almost certainly be applied to the study of seaweed genetic mapping, marker assisted breeding and genome fingerprinting. Meanwhile, searching for new and valid fingerprinting methods for the study of seaweeds is still important because that search will make fingerprint methods more valid for facilitating marker assistant selection of seaweeds. Improved DNA and RNA extraction methods are also needed. Establishing fingerprints and genetic background of seaweed cultivars is crucial in providing information for improving preservation of seaweeds. Finally, constructing the physical map or gene library and conducting QTL analysis for commercial seaweeds is necessary. It is worthwhile to target the gene and analyze function during the seaweed's growth, development and reproduction and eventually apply markers and DNA fingerprint techniques to serve seaweed breeding and propagation.

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6

Genes Involved in the Compensatory Growth Induced by Refeeding in European Sea Bass (*Dicentrarchus labrax*)

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6.1 INTRODUCTION

Fish alternate between feeding and fasting periods during their annual cycle as a consequence of temporal and spatial food availability in the aquatic environment. They are well adapted to survive for long periods without food, and metabolic depression seems to be an important strategy in response to periods of food scarcity (Cook *et al*., 2000; O'Connor *et al*., 2000). Extensive body energy reserves may be lost during such periods as the fish metabolize their own tissues to meet critical energy, reflected by a decrease in growth rate and in morphological indexes such as condition factor (K), an indicator of body shape. Fasting-associated growth retardation is completely overcome, or at least reduced, if an abundant food supply becomes available after a prolonged period of food shortage. Then, fish display a rapid growth spurt known as compensatory growth (Mackenzie *et al*., 1998). In rare cases, fish displaying this characteristic are able to surpass in size those individuals that did not undergo nutritional restriction (Nikki *et al*., 2004; Hayward *et al*., 1997).

Compensatory growth is of interest in aquaculture, because by understanding these dynamics, feeding schedules can be designed that

improve growth rates (Hayward *et al*., 1997). There are several descriptions of compensatory growth in fishes, mostly in salmonids, but only few generalizations have emerged and the mechanisms involved in such rapid recovery from fasting are still not fully understood. Such mechanisms have principally been searched for at the level of total fish growth, where a number of circulating hormones are thought to be involved (Fauconneau *et al*., 2000). Some attention has also been paid to muscle growth and to locally produced paracrine/autocrine factors (Chauvigné *et al*., 2003; Johansen and Overturf, 2006), which can profoundly affect tissue growth and development.

On the other hand, whereas much is known about the physiological and biochemical response of fish to starvation, only little information is available at the molecular level. In particular, we know very little about the response of genes at the transcriptional level to starvation and refeeding, although this knowledge could supply greater insight into critical biochemical pathways that control vertebrate muscle growth, and is also relevant to understanding the mechanisms by which the expression of such genes is regulated.

Our research focuses on identifying candidate genes whose expression contributes to the compensatory growth induced by refeeding in sea bass (*Dicentrarchus labrax*). This species is of great interest for Mediterranean aquaculture as it is an excellent food fish with high commercial value. Moreover, it also represents a good model organism for muscle growth studies as, unlike traditional fish models such as zebrafish and mekada, it shows extensive postlarval muscle hyperplasia, which contributes to its large adult size.

Accordingly, in our studies (reviewed here) we have isolated the complete cDNAs coding for some physiologically relevant proteins in this teleost, and then have assessed the impact of chronic feed deprivation and subsequent refeeding on their mRNA levels in different tissues, with the aim to relate these expression levels to feeding status.

6.2 POSITIVE AND NEGATIVE GROWTH FACTORS

It is already known that the brain processes and responds to nutritional stimuli with appropriate modifications in growth through hormonally mediated pathways (Duan, 1998). A central step in this endocrine pathway is the growth hormone (GH) - insulin-like growth factors' (GH-IGFs) axis. The majority of the growth promoting effects of the growth hormone is believed to be mediated by insulin-like growth factor-I

(Leroith *et al*., 2001; Duan, 1998; Moriyama *et al*., 2000), which is structurally related to IGF-II (Shamblott and Chen, 1992). Both substances were termed 'insulin-like' because of their ability to stimulate glucose uptake into muscle and fat cells (Randle, 1954). Thus, it is not surprising that they share an approximately 50% amino acid identity with insulin. IGF-I and IGF-II are ubiquitous small peptides, well known for stimulating many anabolic responses on a variety of target cells in both endocrine and autocrine/paracrine fashions (Jones and Clemmons, 1995). IGFs (also known as somatomedins) are unique among the growth factors, having the property of stimulating both proliferation and differentiation of muscle precursor cells (myoblasts or satellite cells) as well as myocyte hypertrophy during muscle regeneration (Florini *et al*., 1996; Musaro *et al*., 1999).

Many important biological actions of IGF-I and IGF-II that participate at different levels in the regulation of vertebrate growth, development, and differentiation have been extensively documented. IGF-I functions as postnatal and adult life GH-dependent growth factor, whereas IGF-II shows controversial GH dependence and is considered a major embryonic, foetal, and early neonatal life growth factor (Sara and Hall, 1990; Levy *et al*., 1992). The physiological role of IGF-I and -II was confirmed in mice, where a significant growth retardation was observed in pups in which the gene for IGF-II was disrupted or knocked out (De Chiara *et al*., 1991), but a null mutation for the IGF-I gene produced muscles with a reduced number of myofibers (Powell-Braxton *et al*., 1993). In contrast, a direct infusion of IGF-I into the tibialis anterior muscles of adult rats led to increased total muscle protein and DNA content, demonstrating skeletal muscle hypertrophy concomitant with satellite cell activation (Adams and McCue, 1998).

Functional studies in fish indicate that the biological potency of IGFs is remarkably conserved throughout evolution. IGFs constitute a central endocrine link in the chain of hormones that regulate fish growth. Skeletal muscle is an important target tissue for these hormones: IGF-I acts as a potent positive regulator of muscle growth (Florini *et al*., 1996) and IGF-II affects the progression of the muscle cells through the second half of G1 and entry into S phase within the cell cycle (Hill, 1992).

Skeletal muscle constitutes the largest fraction of fish body weight, up to 60% in some species, and it is nutritionally sensitive (von der Decken and Lied, 1992). Unlike mammals and birds, fish muscle continues to grow, albeit with possible variations in the growth rate, throughout a much greater period of the life cycle (Johnston, 1999). In birds and mammals, the increase in the number of muscle fibers

(hyperplasia) stops at, or shortly after birth (Campion, 1984) and further muscle growth is mainly caused by outgrowth of existing fibers (hypertrophy). In most teleosts, the addition of new fibers continues to be an important contribution to normal muscle growth well into adulthood (Weatherley *et al*., 1988) and it also appears that the importance of the IGF system in muscle is maintained at a higher level for much longer throughout adult life (Méndez *et al*., 2001), suggesting a special role of these growth factors in fish physiology.

IGF-I and II transcripts have been detected in many fish tissues, with liver containing the greatest amount (Shamblott and Chen, 1992, 1993). The evidence that IGF-I and IGF-II mRNAs are also expressed in other tissues (Duan *et al*., 1993) and the presence of receptors in these tissues suggests that, in fish, paracrine and autocrine actions of IGFs are also involved in organ-specific functions.

Nutrient availability and a multitude of environmental factors modulate the rate of muscle growth in fish. The proliferation activity of myogenic precursor cells *in vitro* and *in vivo* depends largely on the feeding status in fish (Brodeur *et al*., 2002; Fauconneau and Paboeuf, 2000), suggesting that the cellular aspects of muscle growth are influenced by the feeding status of the fishes. In physiological as well as in experimental fasting, both hypertrophic and hyperplasic components of muscle growth are stopped (Kiessling *et al*., 1990). This is thought to be associated with changes in the expression of growth factors in muscle. Furthermore, in various fish species, it has been shown that hepatic (Duan and Plisetskaya, 1993; Matthews *et al*., 1997) and muscular (Chauvigné *et al*., 2003) IGF-I and IGF-II mRNA levels depend on feeding status.

The complex processes of myogenesis and muscle growth are precisely regulated by a number of extrinsic regulators. Among these are growth factors such as fibroblast growth factor 6 and myostatin (MSTN), a member of the transforming growth factor-β (TGF-β) superfamily. Among the members of the fibroblast growth factor family, which represent *in vivo* moderators of critical phases of muscle development, FGF6 might play a specific role since it has an expression profile essentially restricted to developing and adult skeletal muscle (de Lapeyriere *et al*., 1993; Han *et al*., 1993; Pizette *et al*., 1996). The prolonged muscle hyperplasia in fish is associated with a continuous expression of FGF6 up to the adult stage (Rescan, 1998), unlike in mammals where its expression is restricted to the pre- and perinatal period. The lasting expression of FGF6 in fish well into adulthood

suggests that it participates in the continuous generation of muscle fibres within the myotomic muscle of postlarval animals.

Whereas FGF6 is involved both in the proliferation and in the differentiation of the myogenic lineage (Armand *et al*., 2003) by stimulating myoblast proliferation, myostatin, a member of the TGF-β family, is a negative factor and inhibits myoblast proliferation. The TGF-β superfamily comprises multiple growth/differentiation factors (GDF) that play important roles in the regulation of growth and development of many diverse tissues, including skeletal muscle (Zimmerman and Padgett, 2000). In a study by McPherron *et al*. (1997) a growth differentiation factor (GDF-8) was disrupted in mice and the GDF-8 knock-out mice showed a marked increase in skeletal muscle mass due to muscle fibre hyperplasia, but also hypertrophy. As GDF-8 seemed to function as an inhibitor of muscle growth, it was renamed myostatin (MSTN). MSTN is a negative regulator of the number of myoblasts and, hence, number of fibres, as demonstrated by the fact that a short deletion in the third exon of the MSTN gene was found to be the reason for the double-muscling phenomenon in cattle (Grobet *et al*., 1997).

MSTN seems to act predominantly on muscle growth. It is the main factor involved in depressing both the number of myoblasts that reach terminal differentiation divisions and, to some extent, the degree of fibre enlargement. As a consequence, the lack of MSTN results in increased muscle mass (Bass *et al*., 1999). Since the initial characterization of MSTN (McPherron *et al*., 1997) and FGF6 in mouse, dozens of additional cDNAs have been isolated in different vertebrate species, whereas orthologues from commercially important fish species have only been identified in a few species. Furthermore, only little is known about the participation of these genes in modulating muscle growth in response to different feeding regimens.

6.3 APPETITE STIMULATING SIGNALS

Feeding and satiation in fish, as in other vertebrates, appears to be regulated via complex mechanisms involving elaborate interactions between the brain and peripheral signals (De Pedro and Bjornsson, 2001). Even in mammals, where such mechanisms have been studied for decades, they are not yet clearly defined (Stanley *et al*., 2005; Volkoff *et al*., 2005).

A current model proposes there is a central feeding system that has the overall control over food intake. This system involves specific nuclei

of the hypothalamus and processes information received from two major peripheral systems: a short-term system, also called the peripheral satiety system, which transmits signals of appetite and satiation, and a long-term system, which provides information regarding the status of body energy stores (Jensen, 2001). The hypothalamus produces neuropeptides such as corticotropin-releasing factor, melanin-concentrating hormone, and neuropeptide Y that stimulate or inhibit feeding (Schwartz *et al.,* 2000; Woods, 2005). The peripheral signals include short-term or 'satiety signals', such as cholecystokinin, peptide YY, and glucagon-like peptide-1, which originate primarily from the gastrointestinal tract, and long-term or 'adiposity signals', such as insulin, secreted from pancreatic B cells, and leptin, secreted from adipocytes (Konturek *et al.,* 2003; Halford *et al.,* 2004; Strader and Woods, 2005; Volkoff *et al.,* 2005). All the peripheral signals affect the brain, interacting with specific receptors on the afferent fibers of the vagus nerve (e.g., cholecystokinin) or by reaching the brain directly via the blood (e.g., amylin and leptin). Furthermore, most of the gastrointestinal peptides are also synthesized in the brain and thus considered to be both a central and peripheral signal, demonstrating the complex nature of this regulation, which includes both central and peripheral factors that all seem to communicate with each other (Konturek *et al.,* 2003; Halford *et al.,* 2004; Volkoff, 2006).

A key element of this communication process is the hunger-inducing hormone ghrelin, which is believed to convey information about nutrient availability from the stomach to the brain (van der Lely *et al.,* 2004; Cummings *et al.,* 2005). The characterization of ghrelin has made an important contribution to the understanding of hypothalamic control of food intake. Ghrelin, a recently identified 28 amino acid peptide (Kojima *et al.,* 1999), is a potent, peripherally active, appetite-stimulating hormone and is predominantly secreted by the stomach (Sakata *et al.,* 2002), but is also synthesized in the brain (Cowley, 2003). This unique acylated peptide hormone was originally isolated from mammalian stomach tissue and subsequently shown to be an endogenous ligand for the growth hormone secretagogue receptor (GHS-R) (Kojima *et al.,* 1999).

Among the teleosts, ghrelin has been cloned and purified only recently in some species: goldfish, *Carassius auratus* (Unniappan *et al.,* 2002); eel, *Anguilla japonica* (Kaiya *et al.,* 2003a); Mozambique tilapia, *Oreochromis mossambiques* (Kaiya *et al.,* 2003b); Nile tilapia, *Oreochromis niloticus* (Parhar *et al*., 2003); rainbow trout, *Onchorhynchus mykiss* (Kaiya *et al*., 2003c); channel catfish, *Ictalurus punctatus* (Kaiya *et al*., 2005), and sea bream *Acanthopagrus schlegeli* (Yeung *et al.,* 2006). As in

mammals, ghrelin mRNA is mainly expressed in fish stomach/intestine, and moderate levels are detected in the brain (Kaiya *et al.,* 2003c; Parhar *et al.,* 2003). Similarly, growth hormone secretagogue receptor identified recently in pufferfish (Palyha *et al.,* 2000) and black seabream (Chang and Cheng, 2004) shows high levels of expression in the pituitary and the brain, in particular in the hypothalamus (Chang and Cheng, 2004), but also in a variety of other tissues, indicating the potential for both systemic (endocrine) and local (autocrine/paracrine) action of this hormone system (Korbonitis *et al.,* 2004).

Accumulating evidence indicates that ghrelin has a major role in feeding, metabolism, and reproduction in fish. It stimulates food intake (Unniappan *et al*., 2004), thereby providing essential nutrients for fish growth, and required energy for metabolism and reproduction. Fasting-induced increases in gut and hypothalamus ghrelin mRNA levels with significant increases in serum ghrelin levels, reported in teleosts (Unniappan *et al*., 2004) provide further support to the orexigenic actions of this peptide. However, the exact role that ghrelin system (i.e., ghrelin and its cognate receptor) plays in fish and its regulatory mechanism are still largely unknown. Indeed, this orexigenic hormone may represent a promising candidate for a functional link between the short-term regulation of feed intake and long-term regulation of growth and metabolism; in order to establish this, however, more studies on fish species are needed (Björnsson *et al.,* 2002).

6.4 DIGESTION ENZYMES

Digestion in fish begins in the stomach instead of the mouth. During fasting the stomach is nearly deplete of gastric juices, while the presence of food produces abundant secretion. As in mammals, the distension produced by food entering the stomach stimulates secretion of pepsinogens, the inactive precursors of the pepsins, together with hydrochloric acid, which are the two main components of the gastric juice. In nonmammalian vertebrates they are generally both secreted by the same type of cells, the oxynticopeptic cells, which are clustered in glands in the gastric mucosa (Helander *et al*., 1981). The pepsins belong to the family of the aspartic proteinases. These enzymes are in charge of the initial and partial hydrolysis of the dietary proteins, a process that is subsequently completed in the intestine by the combined action of trypsin and chymotrypsin (Lo and Weng, 2006). There are five known groups of pepsinogens (zymogens): pepsinogens A, B, and F, progastricsin (also

known as pepsinogen C), and prochymosin. Upon ingestion of food, the zymogens are converted in the acidic environment of the gastric lumen into their active forms: pepsins A, B, and F, gastricsin, and chymosin, respectively (Kageyama, 2002). The release of the activation segment (the propart of pepsinogen), whose length ranges from 35 to 47 residues among pepsinogens) from the N-terminal part of pepsinogen, represents the major activation process. The primary structures and enzymatic properties of the activated forms of these five types of pepsinogens are different (Kageyama, 2002).

The pepsins exhibit optimal activity at extremely low pH values (Fruton, 1970; Dunn *et al*., 1987), but are quickly denatured when the pH values exceeds 5.5, protecting in this manner the host tissue from self-autolysis. The occurrence of several pepsinogen groups seems to be closely related to ontogeny. Progastricsin and both pepsinogen A and pepsinogen B are predominantly found in the gastric mucosa of adult vertebrates, while prochymosin and pepsinogen F are present only in neonatal mucosa (Richter *et al*., 1998). The differences in enzymatic properties of these groups are apparently adapted to the digestion of a variety of substrates such as milk protein during neonatal stages (Foltmann, 1992), although the developmental changes in the appearance of various pepsinogens may be induced by intrinsic factors such as hormones.

The gastric zymogens have been isolated and sequenced, at either the protein or the DNA level, from a wide range of vertebrates, including mammals (Tang, 2004 a,b; Foltmann and Szesci, 2004), avians (Bohak, 1969; Donta and Vunakis, 1970), reptiles such as turtles (*Trionyx sinensis*) (Hirasawa *et al*., 1996), and snake (*Trimeresurus flavoviridis*) (Yonezawa *et al*., 2000), and amphibians (Yakabe *et al*., 1991). In fish the amino acid sequences of pepsinogens have also been reported in several species, including trout (*Salmo gardneri*) (Twining *et al*., 1983), tuna (*Thynuus orientalis*) (Tanji *et al*., 1996), Atlantic cod (*Gadus morhua*) (Gildberg *et al*., 1990), shark (*Centroscymunus coelolepis*) (Nguyen *et al*., 1998), Antartic fish (*Trematomus bernacchii*) (Carginale *et al*., 2004), tilapia (*Oreochromis mossambicus*) (Lo and Weng, 2006), and sea bream (*Sparus latus*) (Zhou *et al*., 2007).

However, only few studies have examined the gene expression of such enzymes in fish (Douglas *et al*., 1999; Gawlicka *et al*., 2001; Darias *et al*., 2005; Murray *et al*., 2006) and all focused on the developing larvae. Only few studies (Bobe and Goetz, 2001) are related to gene expression of a gastric enzyme (progastricsin) in adult fish.

6.5 MOLECULAR CHARACTERIZATION OF TARGET GENES

At the beginning of our research, the coding sequences of IGF-I, IGF-II, MSTN, FGF6, Ghrelin and Progastricsin were not available in public databases for *D. labrax*; in fact, despite the high commercial interest in this animal, information on its genome, transcriptome, or proteome is minimal. Considering this lack of information, we aimed first to obtain the open reading frames of each gene. A BlastN search (http://www.ncbi.nlm.nih.gov/BLAST/) was performed on the complete, nonredundant Genbank nucleotide database for orthologues of IGF-I, IGF-II, MSTN, FGF6, Ghrelin and Progastricsin in other fish species. A multiple sequence nucleotide alignment was then carried out on the coding sequences found for each gene, and a strategy based on regions of strong nucleotide conservation was used to design the primers. In the case of ghrelin for example, primer design was based on the multiple alignment of three teleost ghrelin coding sequences available on the NCBI Genbank database: *Oreochromis mossambicus*, *Oreochromis niloticus* and *Acanthopagrus schlegelii*. These presented several conserved regions within the sequence where primers could be reasonably designed (Terova *et al*., 2008).

Total RNA was extracted from different sea bass tissues. The quantity of the RNA was calculated using spectrophotometry. The integrity and relative quantity of RNA was checked by electrophoresis. After extraction, an aliquot of total RNA was reverse transcribed into cDNA. PCR amplifications were performed amplifying an aliquot of the resulting cDNA with GoTaq Polymerase. The annealing temperatures depended on the melting temperatures of the primer set used. An aliquot of each sample was then electrophoresed and bands were detected by ethidium bromide staining. The PCR products from IGF-I, IGF-II, MSTN, FGF6, Ghrelin and Progastricsin primer amplifications were then cloned and subsequently sequenced.

Several cDNA fragments were obtained following this cloning strategy. Then, by connecting the sequences of the partially overlapping clones, partial coding sequences (~ 250 bp) of each gene were determined. The full-length cDNAs of each gene were subsequently isolated by 5'- and 3'- RACE and deposited in GenBank under the accession no. AY800248 for IGF-I, AY839105 for IGF-II, AY839106, for MSTN, AY831723 for FGF6, DQ665912, for Ghrelin, and EF690282 for progastriscsin.

6.5.1 IGF-I and IGF-II

The deduced amino acid sequence of the sea bass IGF-I (accession no. AY800248) showed a high sequence identity with other teleosts and mammalian species: 94% identity at the amino acid level with the *Mugil cephalus* sequence, 88% with *Oncorhynchus kisutch* (coho salmon) (Cao *et al*. 1989), 88% with *Oncorhynchus mykiss* (rainbow trout) (Shamblott & Chen 1992), 80% with *Carassius auratus*, 58% with *Sus scrofa,* and 63% with its human counterpart.

Sea bass IGF-II (accession no. AY839105) also showed a high sequence identity with that of other teleosts and mammals: 93% sequence identity at the amino acid level with *Lates calcarifer* (Collet *et al*. 1997), 85% with *Oreochromis mossambicus* (Chen *et al*. 1997), 84% with *Oncorhynchus mykiss* (accession no. M95184), 71% with *Ictalurus punctatus* (Peterson *et al*. 2004), 51% with *Mus musculus,* and 46% with its human counterpart. The structure of other teleost IGFs seems to be highly conserved through evolution, too. For example, rainbow trout IGF-I and IGF-II proteins (61% homology between each other) share a 80% and 65% homology with human IGF-I and IGF-II, respectively (Shamblott & Chen 1992), whereas salmon and human IGF-I differ only in 14 out of 70 amino acids (Cao *et al*. 1989).

Such high levels of evolutionary constraints for different IGF sequences in fish, which represents the largest and most diverse group of vertebrates, suggest the importance of these peptides for growth and development. In addition, the high level of sequence conservation among all known IGFs orthologues suggests that also the structure/function relationship of each protein is equally well conserved among vertebrates as well, although this does not necessarily imply that the function of IGFs is entirely conserved across the vertebrate class as species-specific differences are also likely to exist.

6.5.2. Myostatin and FGF6

The sea bass FGF6 (accession no. AY831723), showed a high sequence identity with other teleost and mammalian FGF6s: an 87% sequence identity at the amino acid level with the FGF6 sequence in O*ncorhynchus mykis* (Rescan, 1998), one of 80% in D*anio rerio* (accession no. AF516334), of 65% in *Mus musculus* (accession no. M92416), and of 62% with its human counterpart (accession no. AY581424).

The deduced amino acid sequence of the sea bass MSTN showed also a high sequence identity with other teleosts and mammalian species: a 97% identity at the amino acid level with the sequence in *Morone*

chrysops (Rodgers *et al*., 2001), one of 94% in S*parus aurata* (accession no. AF2584489), one of 91% in tilapia (*Oreochromis mossambicus*) (Rodgers *et al*., 2001), one of 82% with both MSTN isoforms of O*ncorhynchus mykiss* (Rescan *et al*., 2001), one of 63% in *Canis familiaris*, (accession no. AY367768), one of 63% in *Sus scrofa* (accession no. AY448008) and one of 63% in its human counterpart (accession no. AF104922).

The sea bass MSTN, in accordance with all of the previously described MSTNs orthologues, possess four cysteine residues in addition to the nine cysteines common to all TGF-β family members (Rodgers and Weber, 2001). The nine invariant cysteine residues found in all TGF-β family members contribute to the formation of the characteristic cysteine knot through intramolecular disulphide bonds, while it is unknown whether the four additional cysteines found in MSTN orthologues participate in disulphide linkages (Rodgers *et al*., 2001). The sea bass MSTN protein sequence (376 amino acids) is shorter than that of sea bream (385 amino acids, accession no. AF258448), due to the absence of the polyglutamine stretch (12 residues) in the amino-terminal region (Terova *et al*., 2006), but not longer than in other known vertebrate MSTN orthologues (377 amino acids in rat MSTN and 376 in zebrafish and all the others).

6.5.3 Ghrelin

Sea bass ghrelin (accesion no. DQ665912) showed the highest sequence homology with teleosts: (seabream: 77%; tilapia and Nile tilapia: 71%; rainbow trout: 57%) and avian species (chicken: 52%), and lower homology with amphibians and mammals (bullfrog: 40%; rat, human: 45%). In the sea bass and other non-mammalian preproghrelin sequences, is not present a conserved sequence similar to obestatin. This 23 amino acid peptide isolated recently in mammals (Zhang *et al*., 2005), has been proposed as a hormone that antagonizes the appetite regulatory effects of ghrelin, but no report has been published so far on the identification of a teleostean obestatin. Noteworthy in the sequence of sea bass mature ghrelin peptide is that the C-terminal portion rather than its N-terminal end shows high variability (Terova *et al*., 2008). At the N-terminal end of the mature ghrelin peptide there is strong conservation of the first seven amino acids, including a serine residue at the position 3, which is the site of fatty acylation with *n*-octonoic or *n*-decanoic acid, an essential modification for receptor binding of ghrelin (Muccioli *et al.,* 2001). The N-terminal region is the biologically active segment of the

ghrelin and the first four amino acids "GSSF" are considered to be the "active core" of the ghrelin peptide in mammals (Bednarek *et al.,* 2000).

6.5.4 Progastricsin

For progastricsin (accession no. EF690282), our study has ventured beyond the messenger to characterize the gene in *Dicentrarchus labrax* (Terova *et al*., 2007b). To date, the structure of this gene has been studied only in humans (Hayano and Sogawa, 1988), rat (Ishihara *et al*., 1989) and chicken (Hayashi *et al*., 1988). The sea bass progastricsin gene is separated into 9 exons by 8 introns (Figure 6.1), all possessing the canonical GT/AG boundaries (Table 6.1) (Breathnach and Chambon, 1981). The number of exons and the sites of insertion of the introns conincide perfectly with the orthologous genes of humans, rat, and chicken, while the length of the intron sequences is very different. In fact, the human pepsinogen C gene occupies approximately 10.7 kb of the genomic DNA while that of sea bass is much shorter, occupying only 3.1 kb. The organization 9 exons + 8 introns is not exclusive to the progastricsin gene; in fact, it is common to all pepsinogens, including all the aspartic proteases, suggesting that these proteins evolved from a common ancestral gene (Takahashi, 1992).

Table 6.1: Sea bass (Dicentrarchus labrax) progastricin exon/intron boundaries, length and position of introns and exons, calculated considering +1 the position of the nucleotide A of the start (ATG) codon

Exons				Introns		
No.	**Position**	**Length (bp)**	**5′ splice donor**	**No.**	**Length (bp)**	**3′ splice donor**
1	-36→53	89	ATTCAAGTAAGA	1	134	CCTCAGGGTCCC
2	187→329	142	GCTGATGTAAGA	2	125	TCCCAGACCACC
3	454→572	118	CCTGCAGTGAGT	3	342	TTGCAGACACAC
4	914→1033	119	GTCACTGTGAGT	4	351	TCATAGGTTGCC
5	1384→1584	200	TTCCAGGTATAA	5	153	GTTTAGGAATGG
6	1737→1854	117	TGAAGGGTGTGC	6	138	TTTCAGATTCCA
7	1992→2140	148	GGAGCGGTAAGA	7	279	CTCCAGTATATG
8	2419→2518	99	AAACAGGTACGT	8	129	CTCCAGCAATAC
9	2647→3107	460				

Among fish progastricsins, great variations exist in homology. This is different from the situation observed in other vertebrate groups, such as mammals, in which homology among mammalian progastricsins is very high. Sea bass pepsinogen C is, in fact, most homologous to that of

Trematomus bernacchii, showing an approximately 82% in aa identity. Homology with the brook trout (*Salvelinus fontinalis*) and Atlantic cod (*Gadus morhua*) is lower, being 76% in both cases, while homology with winter flounder (*Pseudopleuronectes americanus*) progastricsin is even lower, with merely a 45% aa identity.

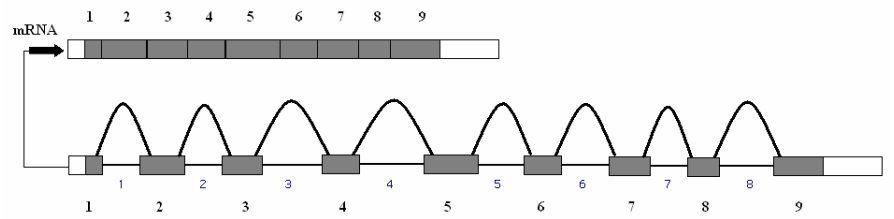


Figure 6.1: Schematic diagram illustrating the intron/exon organization of the of sea bass progastricsin gene. The open boxes indicate 5′ and 3′ untranslated regions. The grey boxes represent the exons, and the thick horizontal lines represent the introns.

As for other aspartic proteinases, the *D. labrax* pepsinogen C includes the prepeptide (signal peptide), the propeptide (activation segment), and the pepsin moiety comprised 16, 31, and 339 amino acids (Terova *et al*., 2007b). The propeptide showed considerable identity with prosegments in other species. More significantly, residues Pro-5, Leu-6, Lys-10, Arg-13, Gly-20, and Leu-22 of the prosegment are conserved in sea bass progastricsin as in nearly all gastric proteinases whose sequences are known. The catalytic cleavage of the propart promotes formation of the active enzyme (Kageyama *et al*., 1989). The catalytic mechanism depends on the presence of two highly conserved, essential aspartyl residues (numbering Asp86 and Asp270 in sea bass) positioned in the center of a deep cleft, forming the active cite and covered by a hairpin loop (flap) protruding from the N-terminal lobe of the molecule. Following propart cleavage at the N-terminal, the flap is dislodged from the substrate cleft, allowing enzyme-substrate interaction to occur (Kageyama *et al*., 1989). Replacements of two aspartates by other residues, as found in the Asp32Ala mutant, inactivates the enzyme (Lin *et al*., 1989).

6.6 PHYLOGENETIC ANALYSIS

To analyze the evolutionary relationship of sea bass IGF-I, IGF-II, MSTN, FGF6, Ghrelin and Progastricsin with respect to other publicly available, related genes in other teleosts and in amphibian, avian, and mammalian species, we reconstructed phylogenetic trees (Figure 6.2-6.6). These

analysis were computed by TREEFINDER, version from October 2005 (Jobb *et al*., 2004). This program computes phylogenetic trees from nucleotide sequences, using the widely accepted Maximum Likelihood method and a novel tree search algorithm. It also accepts incomplete site patterns in a data matrix and can reconstruct trees from partially overlapping sequences. In our case, 1000 bootstrap replicates were performed (expressed in % on the branch nodes) for each analyzed gene.

The results showed that IGF-I is grouped with high bootstrap support in the branch of other teleosts, such as *Mugil cephalus* (flathead mullet), *Paralichthys olivaceus* (bastard halibut), and O*reochromis mossambicus* (Mozambique tilapia) (Figure 6.2).

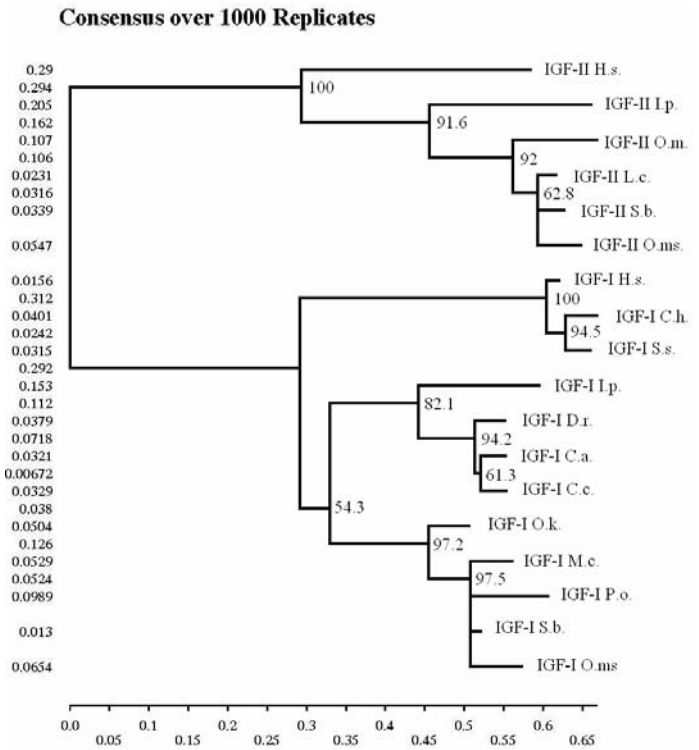


Figure 6.2: Phylogenetic tree comparing the nucleotide sequence of IGF-I and IGF-II in sea bass (Dicentrarchus labrax) (S.b.) with those of other species, i.e., Oreochromis mossambicus (O.ms.), Paralichthys olivaceus (P.o.), Mugil cephalus (M.c.), Oncorhynchus kisutch (O.k.), Cyprinus carpio (C.c.), Carassius auratus (C.a.), Danio rerio (D.r.), Ictalurus punctatus (I.p.), Sus scrofa (S.s.), Capra hircus (C.h.), Homo sapiens (H.s.), Oncorhynchus mykiss (O.m.), Lates calcarifer (L.c.). The scale bar refers to evolutionary distances in substitutions per site. The numbers at tree nodes refer to percentage bootstrap values after 1000 replicates. The numbers on the left hand side of the tree are edge lengths.

The clustering pattern of IGF-II provides evidence that it is grouped into the IGF-II clade of other teleosts, such as O*reochromis mossambicus* (Mozambique tilapia), *Lates calcarifer* (barramundi perch) and *Oncorhynchus mykiss* (rainbow trout) (Figure 6.2).

FGF6 is also grouped with high bootstrap support in the branch of other teleosts, such as *Oncorhyncus mykiss* and *Danio rerio* (Figure 6.3), whereas MSTN is grouped into the MSTN clade of *Tilapia mossambica, Sparus aurata* and *Morone americana* and is not very distinct from other teleosts, such as *Salmo salar*, *Salvelinus alpinus* and *Salvelinus fontinalis* (Figure 6.4).

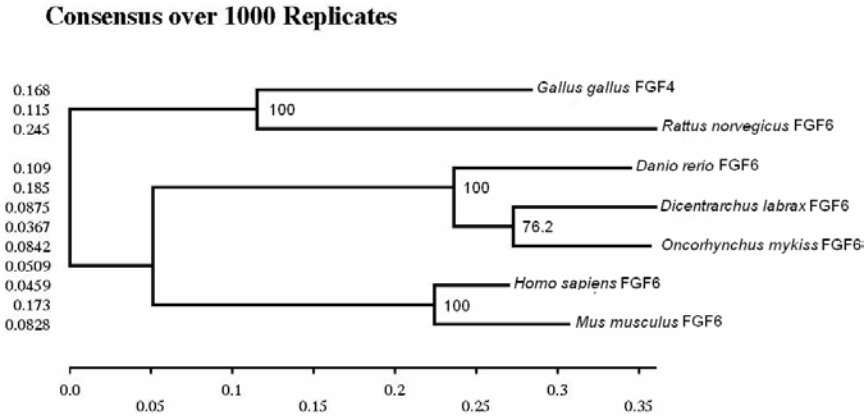


Figure 6.3 Phylogenetic tree comparing the nucleotide sequence of FGF6 in sea bass (Dicentrarchus labrax) (D.l.) with those of other vertebrates.

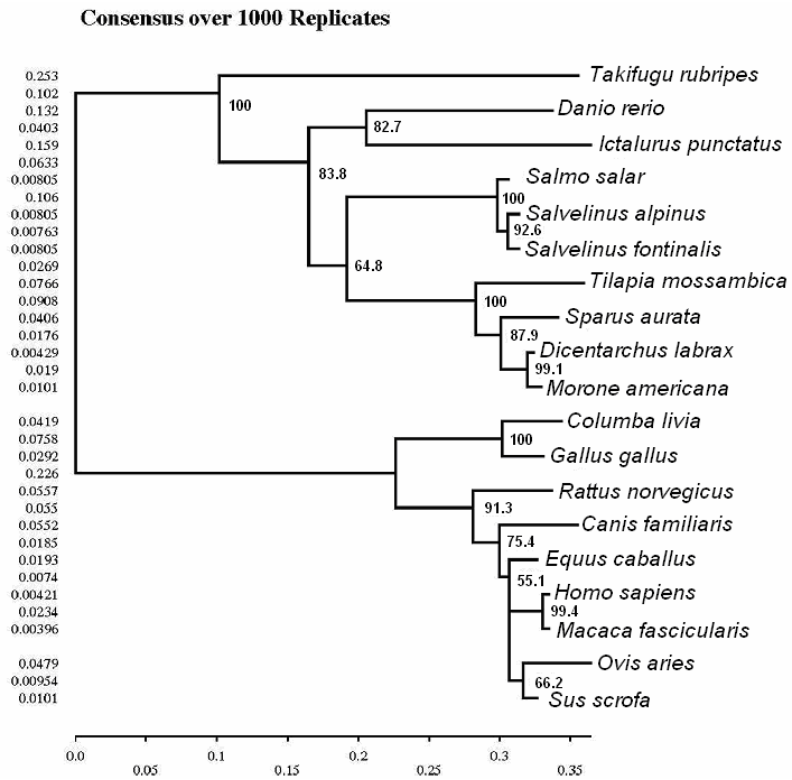


Figure 6.4 Phylogenetic tree comparing the nucleotide sequence of myostatin in sea bass (Dicentrarchus labrax) (D.l.) with those of other teleosts and vertebrates.

Sea bass ghrelin is grouped in the lineage of other teleosts, sharing the highest homology with sea bream ghrelin, whereas the amphibians, avians, and the mammals are grouped into three other distinct lineages (Figure 6.5).

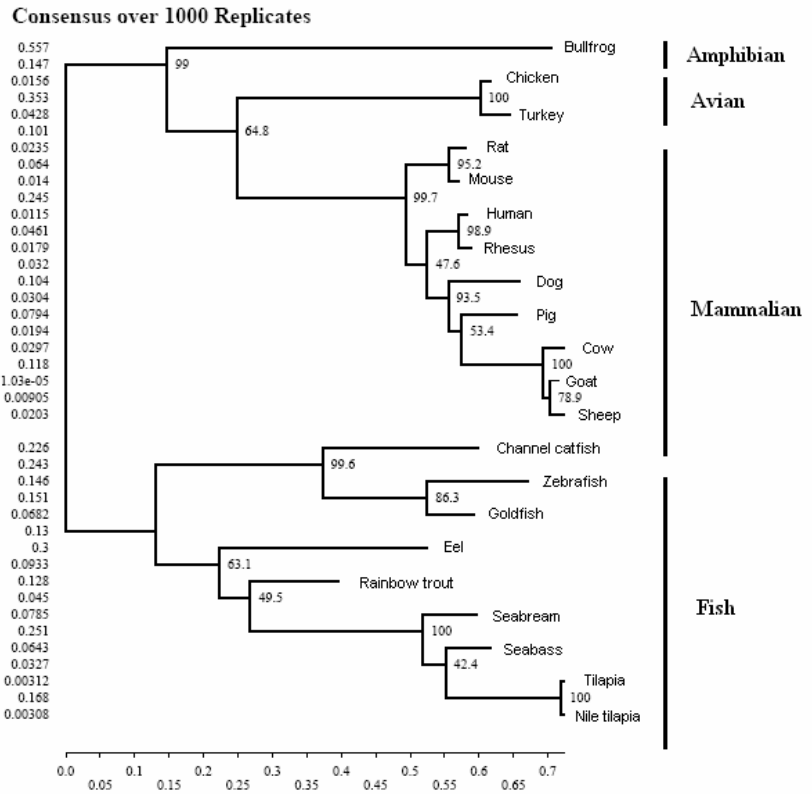


Figure 6.5: Phylogenetic tree comparing the sequence of ghrelin in sea bass (Dicentrarchus labrax) with those of other species. The sequences shown are available from the NCBI GenBank database (accession no: DQ665912 for seabass (Dicentrarchus labrax); AM055940 for zebrafish (Danio rerio); AB196449 for channel catfish (Ictalurus punctatus); AB077764 for tilapia (Oreochromis mossambicus); AB104859 for Nile tilapia (Oreochromis niloticus); AY643808 for seabream (Acanthopagrus schlegelii); AB096919 for rainbow trout (Onchorhynchus mykiss); AB062427 for anguilla (Anguilla japonica); AF454389 for goldfish (Carassius auratus); AB058510 for bullfrog (Rana catesbeiana); AB075215 for chicken (Gallus gallus); AB029434 for human (Homo sapiens) and AB029433 for rat (Rattus norvegicus).

Despite the great variation, fish progastricsins do form a distinctive group as compared to the progastricsins of other vertebrates, as revealed by the phylogenetic analysis shown in (Figure 6.6). Here, pepsinogens C of similar phylogenetic origin are clustered together. The progastricsins of sea bass and Antarctic fish are most homologous to each other while the cod and brook trout progastricsins appear to form a distinctive subgroup among fish pepsinogens C.

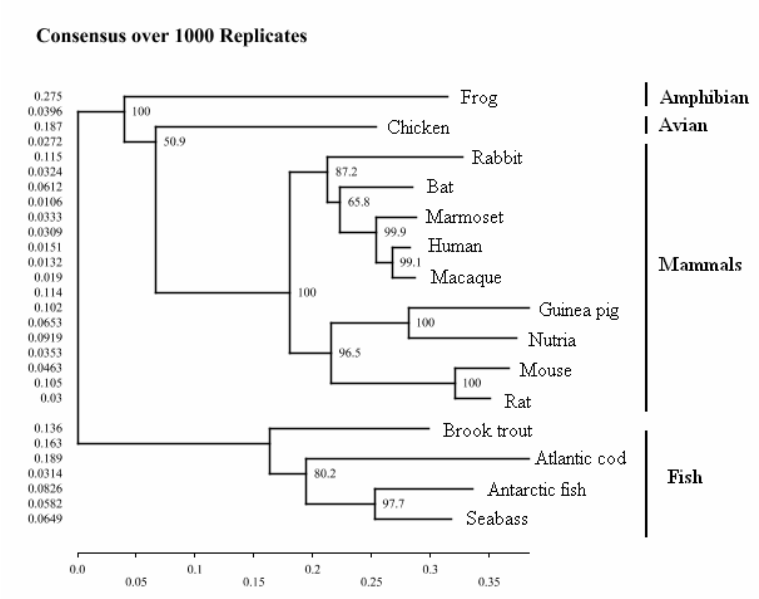


Figure 6.6: Phylogenetic tree comparing the sequence of progastricsin in sea bass (Dicentrarchus labrax) with those of other species. The sequences shown are available from the NCBI GenBank database (accession no: EF690286 for seabass (Dicentrarchus labrax); AJ550952 for Antarctic fish (Trematomus bernacchii); DQ000646 for Atlantic cod (Gadus morhua); AF275939 for brook trout (Salvelinus fontinalis); X04644 for rat (Rattus norvegicus); AK008959 for mouse (Mus musculus); AB188675 for nutria (Myocastor coypus); M88652 for guinea pig (Cavia porcellus); X59754 for Japanese macaque (Macaca fuscata); U75272 for human (Homo sapiens); AB038385 for marmoset (Callithrix jacchus); AB047249 for bat (Rhinolophus ferrumequinum); AB047250 for rabbit (Oryctolagus cuniculus); AB025282 for chicken (Gallus gallus); AB045379 for frog (Xenopus laevis).

The phylogeny of pepsinogens is considered to be useful for estimating that of vertebrates, especially mammals (Narita *et al*., 2006). In particular pepsinogen C is thought to be the most suitable molecular marker since its gene has been shown to exist as a single copy in vertebrates, in contrast to the multiple copies of pepsinogen A genes (Kageyama, 2002). Recently, evidence was obtained indicating that there is only one locus for pepsinogen C in both the human genome and mouse (Puente etal., 2003). Although multiple forms of pepsinogen C have been reported in some animal species (Foltmann and Jensen, 1982; Narita *et al*., 1997; Martin *et al*., 1982) they have been shown to be products of post-translational modifications such as N-glycosylation (Narita *et al*., 1997; Narita *et al*., 2006) and phosphorylation (Jensen and Foltmann 1996).

6.7 DIETARY MANIPULATION OF GENE EXPRESSION

After the isolation of sequences of IGF-I, IGF-II, MSTN, FGF6, Ghrelin and Progastricsin, we assessed by real-time RT-PCR, the impact of chronic feed deprivation and successive refeeding, on mRNA copy number of each target gene, in several tissues of sea bass with the aim to relate their expression levels to the feeding status of the animals, and to determine a possible role of these genes during compensatory growth induced by refeeding.

For this purpose two compensatory growth experiments were carried on. In the first one sea bass juveniles, were reared in three fiberglass raceway tanks, each containing 2.5 m^3 water with 200 fish per tank. The rearing density was below 7 kg/m^3, as the average weight of the fish was 110.8 ± 4.47 g. The tanks were connected to a water recirculation system where salinity was 20 g/L. Other water characteristics were: temperature 21 ± 1°C, pH 8.2, total ammonia <0.2 mg/L, and dissolved oxygen maintained over 99% of the saturation by insufflating pure O_2 to the system. Fish were deprived of food for 4 weeks and then refed with Hendrix-Skretting® Power Excel feed for marine fish (the same type of feed also utilized before fasting). Animals were sampled before fasting (day0), 4 days after the onset of fasting (4dFa), at the end of the fasting period (28dFa) and then sequentially at 1 (1wR), 2 (2wR), and 3 (3wR) weeks following refeeding. They were rapidly anesthetized and body weight and standard body length were measured and used to calculate the condition factor: (K = body weight x 100/ standard body length^3). For the molecular biology analysis tissues were isolated from five fish for each sampling point, frozen immediately, and stored at -80°C until analysis.

In the second experiment the protocol design was slightly modified. The animals were stocked into four tanks of 2 m^3 each, with 35 fish per tank, and allowed to acclimate for 1 month before starting the trial. The tanks were connected to the same aforementioned sea water recirculation system, with strictly controlled water conditions. In the course of the experiment, fish in two tanks were fed to apparent satiety (fed control), whereas fish in the other two tanks were deprived of food for 35 days and then refed to apparent satiety for 21 days with the same type of feed utilized before fasting. Feed consumption (g) in each tank was estimated from the difference between feed delivered into the tank and uneaten feed which was collected from the bottom of the tank. Feed intake was converted to grams of feed consumed per kg body weight (BW) of the

fish per day (Figure 6.7). Five fish from each of the experimental groups were sampled at the following time points: before fasting (day 0), 4 days after fasting, at the end of fasting, and then sequentially at 4, 14, and 21 days following refeeding. Fish were sampled 15 min before the scheduled feeding time. All the fish in the tanks were weighed at the beginning of the experiments, at the end of fasting period and after the refeeding period.

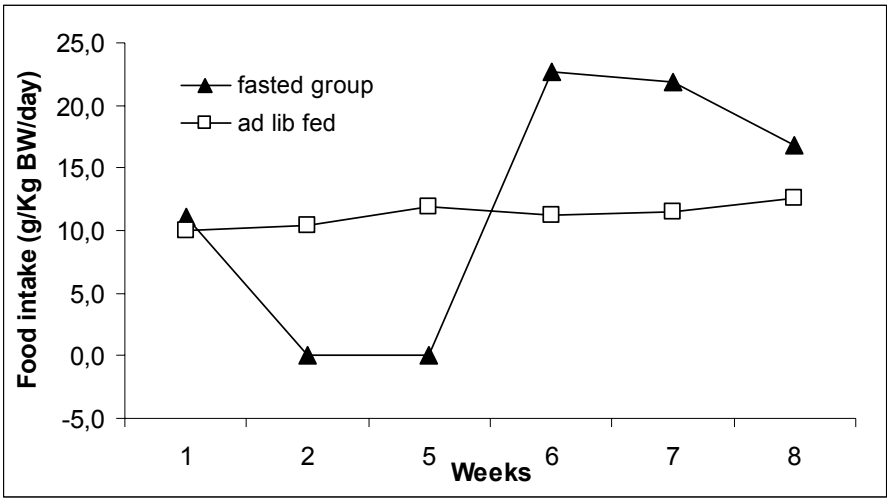


Figure 6.7: Weekly changes in mean daily food intake in the group of sea bass subjected to fasting and refeeding and in that fed ad libitum, during the second experiment.

Total RNA extracted from sea bass tissues was subjected to real-time RT-PCR using the absolute mRNA quantification method. The absolute number of each gene transcript copies was quantified by comparing them with a standard graph constructed using the known copy number of mRNAs of that gene. For example, in the case of progastricsin, a forward and a reverse primer were designed based on the mRNA sequences of the *Dicentrarchus labrax* progastricin we had identified (accession no. EF690282), flanking a region of 400 bp of the progastricsin gene. This primer pair was used to create templates for the *in vitro* transcription of cRNAs for progastricsin: The forward primer was engineered to contain a T7 phage polymerase promoter gene sequence to its 5' end and used together with a reverse primer in a conventional RT-PCR of total sea bass stomach RNA. RT-PCR products were then evaluated on agarose gel

stained with ethidium bromide, cloned, and subsequently sequenced. *In vitro* transcription was performed then, using T7 RNA polymerase.

The molecular weight (MW) of the *in vitro*-transcribed RNA for each gene was calculated according to the following standard formula where progastricsin is reported as an example: progastricsin MW = [129(number of A bases) x 329.2) + 69 (number of U bases) x 306.2) + 66(number of C bases) x 305.2) + 98(number of G bases) x 345.2)] + 159. The result was 157705.2. Spectrophotometry at 260 nm gave a concentration of 528 ng/µl for progastricsin. Therefore, the concentration of the final working solution was 2.02E+12 molecules/µl. The same procedure was applied for the *in vitro* transcription of cRNAs of each target gene.

The cRNAs produced by *in vitro* transcription were then used as quantitative standards in the analysis of experimental samples. Defined amounts of cRNAs at 10-fold dilutions were subjected in triplicate to real-time PCR using one tube-two step TaqMan RT-PCR. The Ct (cycle threshold) values obtained were then used to create the standard curve. Figure 6.8 represent an example of the standard curve established for the progastricsin. The same procedure was applied for obtaining the standard curves of each of the other target genes.

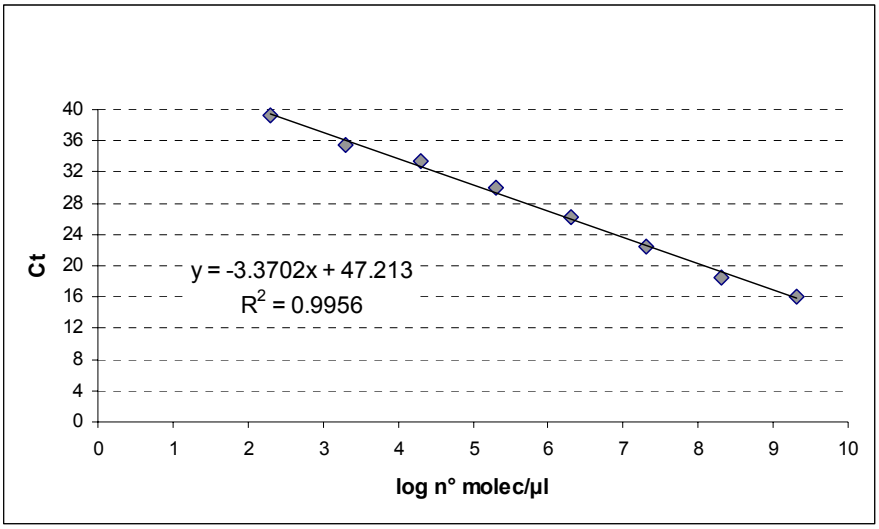


Figure 6.8: Standard curve for progastricsin obtained by amplification of descending 10-fold dilutions of standard cRNAs for progastrcsin.

For the quantitation of each gene transcripts an aliquot of total RNA extracted from the experimental samples was subjected, in parallel to standard cRNAs of that gene, to real-time PCR under the same experimental conditions as used to establish the standard curves. Real-time Assays-by-Design^{SM} PCR primers and gene-specific fluorogenic probes were designed by Applied Biosystems (ABI). TaqMan® PCR was performed on an ABI Thermocycler too. Data from the Taqman® PCR runs were collected with ABI's Sequence Detector Program. The data were statistically compared using one-way analysis of variance (ANOVA). The level of statistical significance was set at $P< 0.05$.

6.7.1 Effect of Dietary Manipulation on Fish Growth Performances

In the first experiment after 28 days of fasting body weight (Figure 6.9a) and condition factor (Figure 6.9b) of fasted fish were significantly lower than the fed controls (Terova *et al*., 2006). During the subsequent refeeding period, previously unfed fish were able to increase body weight sufficiently to overcome weight loss imposed by the 4 week feed restriction. In the second experiment, after 35 days of fasting, the mean body weight of fasted fish (Figure 6.10) was also significantly lower than that of the fed controls. During the subsequent refeeding period, previously unfed fish increased the body weight sufficiently to overcome the weight loss caused by the 5-week feed restriction (Terova *et al*., 2008). Fish that had experienced feed deprivation exhibited significant higher feeding rates than *ad libitum* fed controls during the first two weeks of refeeding. Refeeding of sea bass after 35 days of starvation was marked by hyperphagia as early as the first day. The hyperphagic period was however shorter than the fasting period.

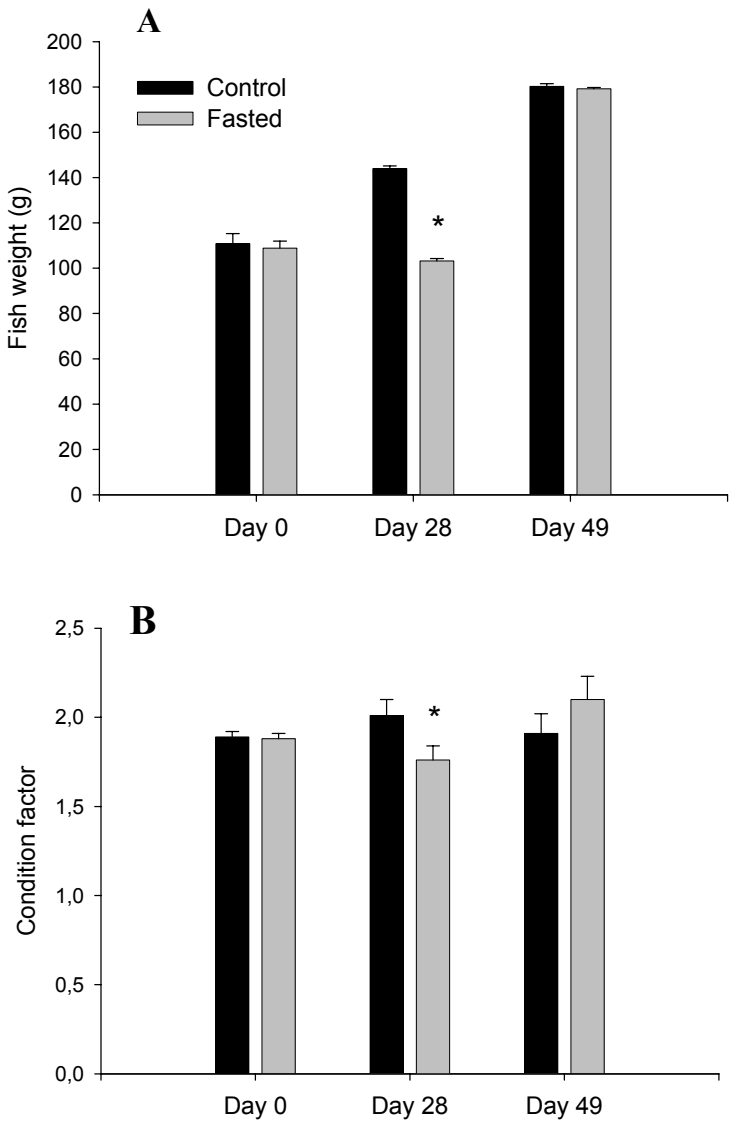


Figure 6.9: Mean body weight (A) and condition factor (B) of sea bass control and fasted group during the first experiment. Fish of the control and fasted group were weighed at the following time points: before fasting (day 0), at the end of fasting (day 28), and then at 21 days following refeeding (day 49). Bars indicate standard error of the mean. Differences were determined by one-way analysis of variance (ANOVA). (*) indicates significantly different means ($P<0.05$).

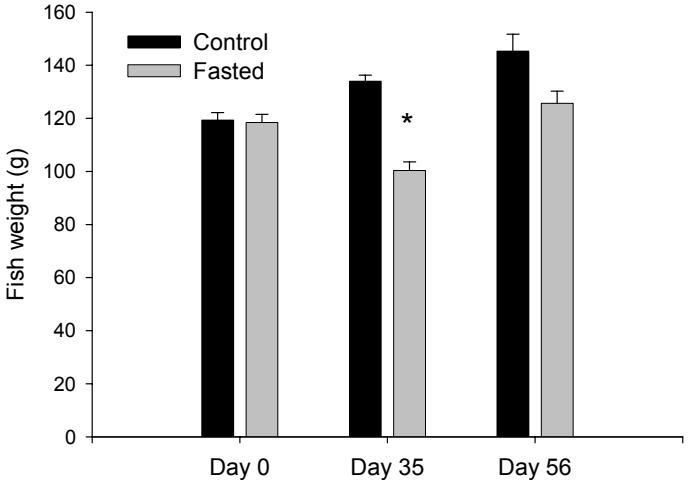
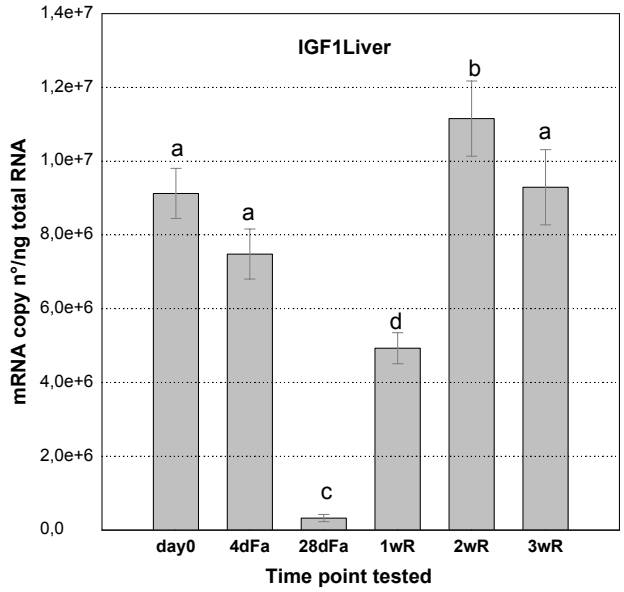


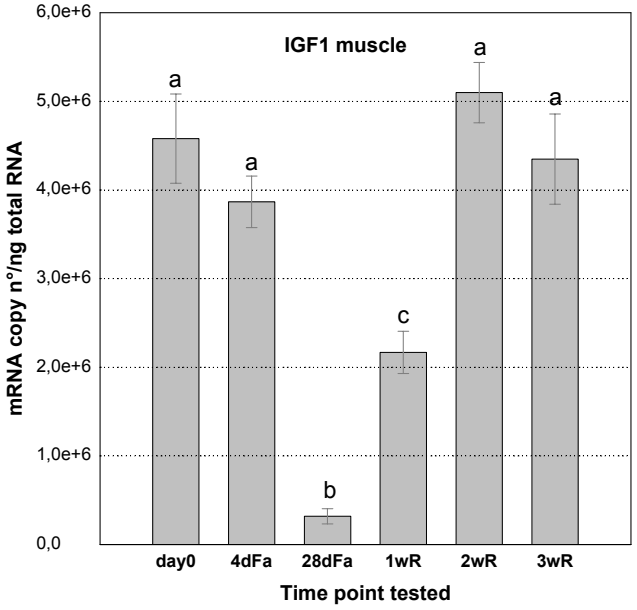
Figure 6.10 Mean body weight of sea bass control and fasted group during the second experiment. Fish of the control and fasted group were weighed at the following time points: before fasting (day 0), at the end of fasting (day 35), and then at 21 days following refeeding (day 56). Bars indicate standard error of the mean. Differences were determined by one-way analysis of variance (ANOVA). (*) indicates significantly different means ($P<0.05$).

6.7.2 Effect of Dietary Manipulation on Gene Expression

6.7.2.1 IGF-I and IGF-II

In our study, IGF-I and IGF-II expression levels measured in liver and muscle of experimental fish confirmed the involvement, albeit with slightly different expression patterns, of both genes in the compensatory growth induced by refeeding (Terova *et al*., 2007a). Fasting of seabass induced a significant decrease in the mRNA copy number of IGF-I and IGF-II in both liver and muscle, indicating that both systemic and local IGF levels were affected (Figure 6.11). A dramatic increase was then observed during the recovery from fasting in the amounts of IGF-I transcript both in liver and muscle; the IGF-II mRNA levels were also up-regulated in both tissues but more gradually than the IGF-I levels. Moreover, 3 weeks of refeeding were not enough for this amount of IGF-II transcript to reach the original values, suggesting that a longer time is necessary for it to completely recover. The response of this growth factor seems to be less affected by nutritional availability.


IGF1Liver
mRNA copy n°/ng total RNA
1,4e+7
1,2e+7
1,0e+7
8,0e+6
6,0e+6
4,0e+6
2,0e+6
0,0
a
a
c
d
b
a
day0
4dFa
28dFa
1wR
2wR
3wR
Time point tested


IGF1 muscle
mRNA copy n°/ng total RNA
6,0e+6
5,0e+6
4,0e+6
3,0e+6
2,0e+6
1,0e+6
0,0
a
a
b
c
a
a
day0
4dFa
28dFa
1wR
2wR
3wR
Time point tested

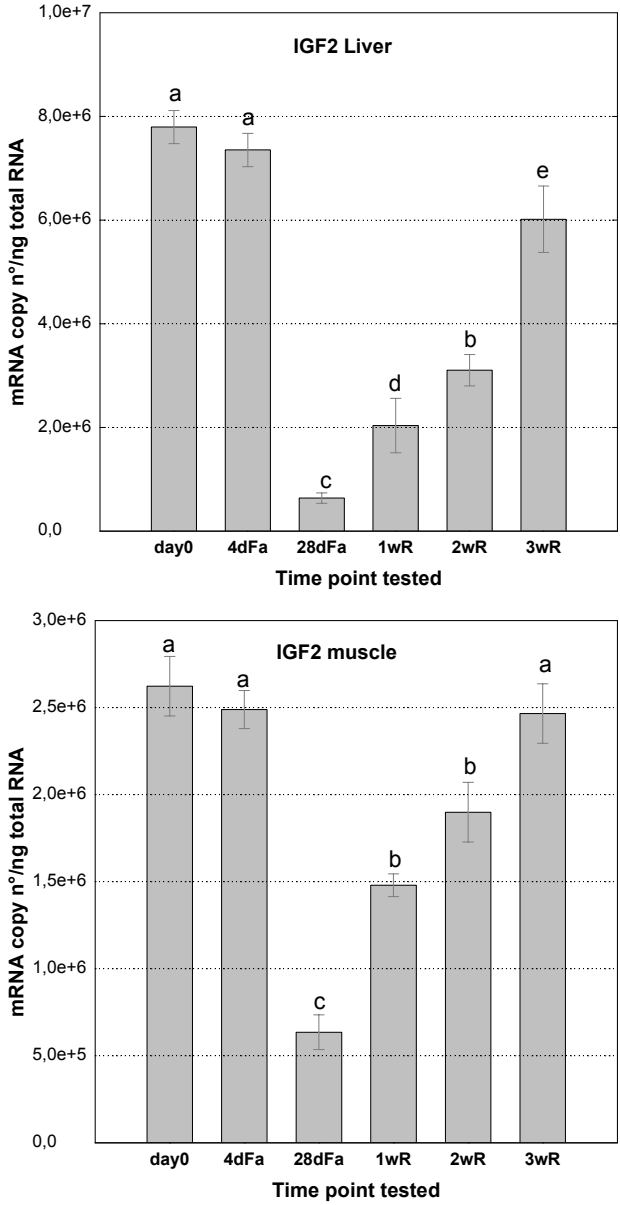


Figure 6.11: IGF-I and IGF-II expression levels measured by real-time PCR in D. labrax liver and muscle. mRNA copy number of each gene was normalized as a ratio to 100 ng total RNA. Fish were sampled before fasting (day0), 4 days after the onset of fasting (4dFa), at the end of the fasting period (28dFa), and then sequentially at 1 (1wR), 2 (2wR), and 3 (3wR) weeks following refeeding. The means of five animals in each group are shown. Bars indicate standard error of the mean. Differences were determined by one-way analysis of variance (ANOVA). Differences between letters indicate significantly different means ($P<0.05$).

The differences in the level of IGF-I and –II may imply different regulation of gene expression and different roles in the growth of sea bass. In various fish species, food restriction decreases the IGF-I mRNA levels in liver (Duan and Plisetskaya, 1993; Matthews *et al*., 1997), whereas refeeding of the starved fish led to a rise in hepatic IGF-I mRNA in coho salmon (Duan *et al*., 1993). In trout, muscular IGF-I and IGF-II mRNA levels have also been found to be lower in starved fish than in refed fish (Chauvignè *et al*., 2003), confirming that autocrine and paracrine IGF expression participates along with the endocrine system in the regulation of tissue growth. In addition, locally produced IGFs are not less functionally important than liver-derived circulating IGF-I. In fact, in mice, autocrine/paracrine IGF-I was able to support normal postnatal growth and development in an experiment of complete inactivation of IGF-I gene in hepatocytes (Yakar *et al*., 1999).

In our study, the highest levels of IGF mRNA were found in the liver, confirming the results of Duan *et al*. (1993) and consistent with the liver as the principal source of endocrine IGF-I in salmon and other vertebrates (Duan, 1998). Furthermore, the amounts of IGF-II transcript found in liver were, in general, higher than those in muscle, in keeping with liver being the major site of IGF-II production as reported by Reinecke and Collet (1998).

IGF-I expression is not restricted to liver; in fact it has also been reported in brain, gill, heart, intestine, kidney, spleen, and testes of several fish species (Biga *et al*., 2004a; Caelers *et al*., 2004; Vong *et al*., 2003). IGF-II is also expressed in a variety of tissues and, in contrast to mammals, in which IGF-II is detected in many foetal tissues but decreases quickly during early postnatal development (Daughaday and Rotwein, 1989), teleostean tissues express substantial amounts of IGF-II later in life (Gabillard *et al*., 2003; Chauvigne *et al*., 2003). Thus, it is possible that IGFs are expressed in a variety of tissues in sea bass, too, suggesting other potential roles in addition to being a positive regulator of muscle growth as originally suggested. Further research is required to elucidate other possible biological functions of IGFs in this species.

6.7.2.2 Myostatin and FGF6

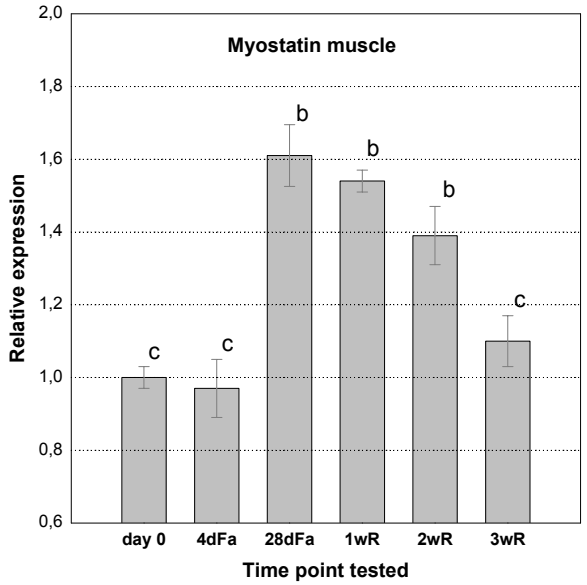
The MSTN abundance levels quantified in our study confirmed the involvement of this gene in the compensatory growth induced by refeeding (Terova *et al*., 2006). A novel finding was the increase in MSTN mRNA levels in the muscle of fasted sea bass, indicating that this autocrine/paracrine growth factor has a role in modulating muscle

growth in response to different feeding regimens. The up-regulation of MSTN during fasting and its decrease during refeeding (Figure 6.12) is in line with previous interpretations of MSTN function demonstrating a negative influence in muscle hypertrophy and hyperplasia (Lee, and McPherron, 1999).

Growth factors such as MSTN have been identified as key peptides in the regulation of myogenesis in vertebrates (McPherron *et al*., 1997; Bass *et al*., 1999). The biological actions of MSTN are extensively documented, and it is believed to repress skeletal muscle growth by inhibiting both muscle cell hypertrophy and hyperplasia (Lee and McPherron, 1999). In accordance with this role, MSTN abundance is higher in the muscle of HIV-infected men with muscle atrophy (wasting) than in healthy men (Gonzales-Cadavid *et al*., 1998), whereas mutations in the MSTN gene in two breeds of cattle, Belgian Blue and Piedmontese, are associated with increased muscle development (Kambadur *et al*., 1997).

If the atrophic actions of mammalian MSTN are indeed conserved in fish, it might be more effective to remove this growth restraint in muscle than to administer growth promoters (Rodgers and Weber, 2001). Therefore, successful manipulation of molecular mechanisms that mediate the effects of MSTN on the growth and development of somatic tissues may have the potential to increase commercial fish production by improving growth efficiency. Results from the quantitative analysis of MSTN abundance levels in response to events such as prolonged fasting, which in turn result in changes in growth rate, are limited in fish and it is difficult to absolutely determine whether MSTN acts as a negative regulator of muscle mass in fish like it does in mammals (Lee and McPherron, 1999).

Recently, Xu *et al*. (2003) produced a transgenic line of zebrafish overexpressing the MSTN prodomain. These transgenic fish exhibited an increased number of skeletal myofibres without any changes in fibre size, suggesting an inhibitory role of MSTN in hyperplastic muscle growth (Xu *et al*., 2003). However regulation of the negative muscle regulator, MSTN appears to be multifactorial, as MSTN levels increased during hindlimb unloading (Carlson *et al*., 1999), decreased with maturation-induced muscle wasting (Rescan *et al*., 2001) and were negatively affected by cortisol treatment (Rodgers *et al*., 2003) and positively (MSTN1) affected by growth hormone treatment (Biga *et al*., 2004b). Our results indicate also a gradual decrease in MSTN abundance during refeeding, suggesting an indirect mechanism of regulation, which future studies may help to clarify.



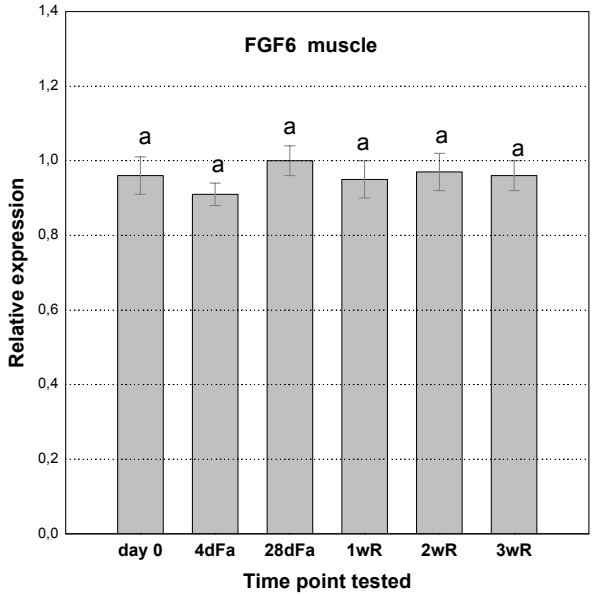


Figure 6.12: Myostatin and FGF6 expression levels measured by real-time PCR in D. labrax muscle. Fish were sampled before fasting (day0), 4 days after the onset of fasting (4dFa), at the end of the fasting period (28dFa), and then sequentially at 1 (1wR), 2 (2wR), and 3 (3wR) weeks following refeeding. The means of three animals in each group are shown. Bars indicate standard error of the mean. Differences were determined by one-way analysis of variance (ANOVA). Differences between letters indicate significantly different means ($P<0.05$).

Unlike MSTN, FGF6 expression pattern did not provide any clue to its function (Terova *et al*., 2006). Muscular FGF6 mRNA levels were not significantly affected in fed, fasted, or refed fish. The response of this growth factor seems to be less affected by the availability of food (Figure 6.12). There are at least three potential explanations for our failure to observe any change in FGF6 transcripts in response to fasting and refeeding: first, it is possible that the duration of fasting in our experiment did not generate the magnitude of nutritional stress necessary to alter FGF6 gene expression, but this is unlikely, as according to the data, fasting was associated with changes in two of the growth indexes examined: body weight and condition factor, which suggests that animals were duly stressed. However, the impact of a more substantial fast on sea bass FGF6 mRNA levels remains to be established in future studies. The second explanation may be that the white muscle FGF6 gene evaluated in our study (accession no. AY831723) was not representative of the isogene that responds to such an experimental design, but additional FGF6 isoforms for the seabass have not yet been characterized. Thirdly, FGF6 may not be directly involved in the modulation of myotomic muscle growth in response to fasting and refeeding, but rather in other processes associated with muscle development and function, in contrast to other FGFs that stimulate satellite cell proliferation (Olwin *et al*., 1994). In this case maybe its role is strictly compensated by other fibroblast growth factors. However, we cannot exclude that FGF6 activity in fasting muscle may be affected by other mechanisms involving post-translational processing, secretion or interaction with binding proteins.

6.7.2.3 Ghrelin

Our studies support a possible role for ghrelin in regulating food intake in sea bass (Terova *et al*., 2008). Our results demonstrate that ghrelin is abundantly expressed in the stomach of this species. Thus, it is also likely that, in sea bass, the stomach is the main expression and production site of ghrelin, as also demonstrated in humans (Ariyasu *et al.,* 2001), catfish (Kaiya *et al.,* 2005) and tilapia (Parhar *et al.,* 2003). Intestinal expression of the ghrelin gene in our species was similar to that reported in catfish (Kaiya *et al.,* 2005) and tilapia (Kaiya *et al.,* 2003b), but relatively low compared with goldfish (Unniappan *et al.,* 2002). This may be due to the fact that goldfish lacks a well-demarcated stomach and therefore the intestinal tract superseded the stomach as the main site of ghrelin gene expression. Brain expression of ghrelin was not significant in sea bass as in previous reports in rainbow trout, channel catfish, Japanese eel,

goldfish and tilapia (Kaiya *et al.,* 2003c; Kaiya *et al.,* 2005; Kaiya *et al*., 2003a; Unniappan *et al*., 2002, Parhar *et al.,* 2003). This finding suggests that ghrelin derived from the brain may be involved in the regulation of feed intake in sea bass, but it does not play a major role such as the stomach derived ghrelin. Weak ghrelin mRNA signal has been observed also in human, rat and mouse brains (Kojima *et al*., 1999; Gnanapavan *et al*., 2002; Guan *et al*., 1997; Tschöp *et al*., 2000).

We found upregulation of ghrelin upon fasting and downregulation during refeeding (Figure 6.13), results that are in line with previous interpretations of ghrelin function and that provide further support for the orexigenic actions of this novel gastrointestinal peptide in fish. Similar fasting-induced changes in stomach ghrelin mRNA expression have been reported in rats (Toshinai *et al.,* 2001), birds (Shousha *et al.,* 2005) and amphibians (Kaiya *et al.,* 2006). In goldfish, starvation induced increases in ghrelin mRNA expression in the gut by day 7, with significant increases in serum ghrelin levels seen as early as days 3 and 5, whereas a postprandial decrease in ghrelin mRNA expression concomitant with a decrease in serum ghrelin levels was also determined (Unniappan *et al.,* 2004).

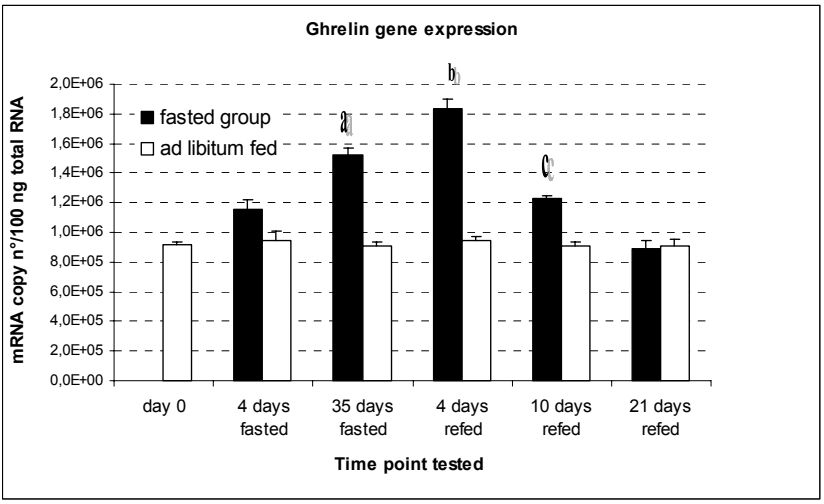


Figure 6.13: Expression levels of ghrelin measured by real-time PCR in D. labrax stomach in the course of the experiment. Ghrelin mRNA copy number was normalized as a ratio to 100 ng total RNA. Fish were sampled before fasting (day 0), 4 days after fasting (4days fasted), at the end of fasting (35 days fasted), and then sequentially at 4, 14 and 21 days following refeeding. The means of five animals in each group are shown. Bars indicate standard error of the mean. Differences were determined by one-way analysis of variance (ANOVA). Different letters indicate significantly different means from controls, for each time point tested ($P<0.05$).

The time course of changes in ghrelin expression in sea bass was relatively low in comparison to mammals and birds, in which stomach ghrelin mRNA levels change significantly in 12-24 h after fasting, but it was similar to the trend observed in amphibians and other fish species. In bullfrog ghrelin mRNA expression in the stomach increased after 10 days of fasting (Kaiya *et al*., 2006), in goldfish after 7 days, while in Nile tilapia, the ghrelin transcript levels did not change even after 7 days of food deprivation (Parhar *et al.,* 2003). The differences in ghrelin responses to fasting may reflect differences in energy metabolism between homeoterms (mammals and birds) and poikiloterms (frogs and fish).

Furthermore ghrelin mRNA levels in our study remained high in the stomach until the fourth day of refeeding and this was an unexpected result. Maybe the extent to which fasted fish display high levels of ghrelin expression on a return to adequate feeding condition is related to the degree to which their energy reserves were depleted during fasting. However future studies are needed to confirm this hypothesis in fish as actually this is the first one to have investigated the ghrelin expression patterns during such a long fasting period.

Fish in our experiment were fed more avidly and grew faster than the controls after the period of fasting, compensating for lost body weight in an apparent attempt to catch up with conspecifics that have had continuous access to food. The mechanisms that determine compensatory growth regulation in fish are not fully understood. What is known from several studies is that growth in fish is regulated by the brain neuroendocrine growth hormone/insulin-like growth factor (GH/IGF) axis (Peter and Marchant, 1995). Ghrelin is undoubtedly a potent stimulator of GH secretion (Unniappan and Peter, 2004; Riley *et al*., 2002; Kaiya *et al*., 2003a,b), which has been shown to play an important role in regulating food intake in fish (Peter, 1995). The increase of food intake (hyperphagia) triggered by the period of fasting in our studies, is a simple but compelling example of food intake regulation. Hyperphagia was the mechanism by which sea bass compensated for their growth loss during fasting. The significant increase in food intake promoted rapid growth, but no improvement in feed efficiency was observed.

We recognize that ghrelin mRNA levels in our studies do not measure physiological effects produced by the protein. Due to this, our hypothesis that ghrelin is an important trigger of the fasting response in the sea bass, probably through its direct effect on GH secretion, is preliminary and will have to be confirmed in the future. Further investigations are also needed to determine, whether the primary effect of ghrelin produced in the sea

bass stomach is to stimulate GH release from the pituitary or to stimulate feeding behaviour directly or indirectly through other hypothalamic factors.

6.7.2.3 Progastricsin

Pepsinogen C in sea bass was abundantly expressed in the stomach, but not in any other tissues tested (Terova et al., 2007b). Thus, it is also likely that, in this species, the stomach is the only expression and production site of this type of pepsinogen. The tissue distribution of progastricsin, however, is not restricted only to the stomach in other vertebrates. High expression of progastricsin in the esophagus is known in the frog (Yakabe *et al*., 1991), while in humans, progastricsin has been identified not only in the gastric mucosa, but also in other tissues, including intestine, pancreas, prostate, and seminal vesicles (Szecsi, 1992). In fish only one study (Bobe and Goetz, 2001), found high levels of progastricsin mRNA in the stomach and also in the postovulatory ovary. This may be due to the fact that in trout the oviduct is not connected to the ovary; therefore, while most fish spawn their eggs soon after ovulation, trout can hold them in the abdominal cavity for at least a week after ovulation. Eggs are then expelled by energetic contractions of the abdominal wall muscles. The presence of progastricsin transcripts in the trout ovary found in this study well past the time of ovulation (10 days) seems to be related to the hydrolysis of the coelomic fluid proteins that remain in the peritoneum after spawning.

To study the nutritional regulation of progastricsin mRNA levels in *D. labrax,* the fish were starved and refed. Progastricsin mRNA levels were downregulated upon fasting and upregulated during refeeding (Figure 6.14) (Terova *et al*., 2007b). These results are in line with previous interpretations of gastricsin function and provide further support for the proteolytic actions of this gastric enzyme, also in fish. Digestion in fish begins in the stomach instead of the mouth. During fasting the stomach is nearly deplete of gastric juices, while the presence of food produces abundant secretion. As in mammals, the distention produced by food entering the stomach stimulates secretion of hydrochloric acid and pepsinogen, which is then hydrolyzed to pepsin. In sea bass, the food is first coated with a mucous layer containing hydrochloric acid and pepsin. This helps prevent dilution by the alkaline seawater that marine fish must continually drink to maintain their osmotic balance. Only one cell type (oxyntic cells, also known as parietal cells) has been found in the gastric glands of fish and it is thought that both hydrochloric acid and

pepsinogen are produced here, while in the mammals, a division of work has evolved: the parietal cells produce acid and the main cells produce pepsinogen.

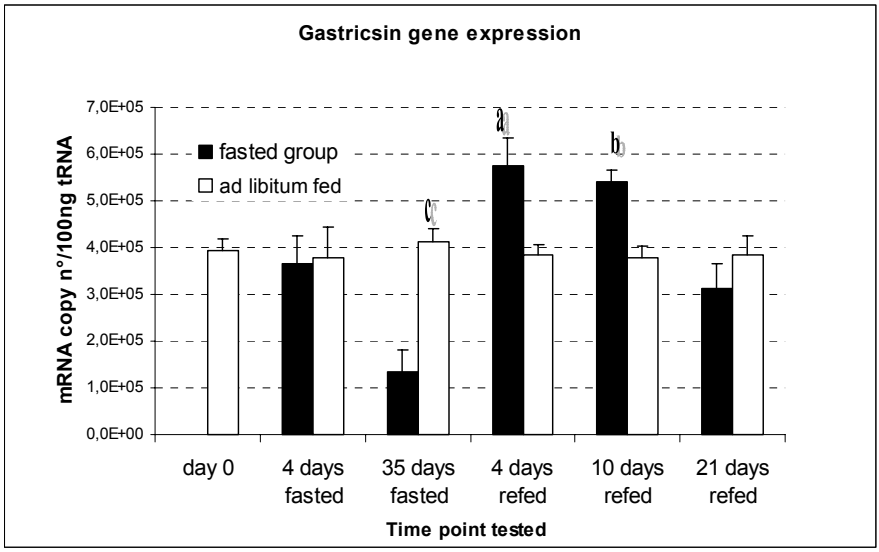


Figure 6.14: Expression levels of gastricsin measured by real-time PCR in D. labrax stomach in the course of the experiment. Gastricsin mRNA copy number was normalized as a ratio to 100 ng total RNA. Fish were sampled before fasting (day 0), 4 days after fasting (4days fasted), at the end of fasting (35 days fasted), and then sequentially at 4, 14 and 21 days following refeeding. The means of five animals in each group are shown. Bars indicate standard error of the mean. Differences were determined by one-way analysis of variance (ANOVA). Different letters indicate significantly different means from controls, for each time point tested ($P<0.05$).

Short-term refeeding (4 days) promoted a remarkable increase in the transcription levels of progastricsin mRNA, which have significantly exceeded the levels of the controls. This result might be explained by the great quantity of protein taken in with the food in the first days of refeeding. In fact refeeding of sea bass after a long starvation period was marked by hyperphagia as early as the first day. It would be interesting to study whether only gastric distension influences the increase in the levels of progastricsin mRNA in fish, or whether the chemical-physical characteristics of the food (as an example the aminoacidic composition) also have a role.

6.9 CONCLUSION

In conclusion, we have isolated the complete coding sequences for IGF-I, IGF-II, MSTN, FGF6, ghrelin and progastricsin in sea bass (*Dicentrarchus labrax*). The nucleic and amino acid sequences of all are highly homologous to the previously identified teleost, avian and mammalian respective orthologs. We have also demonstrated that the nutritional state of the animal influences the levels of expression of IGF-I, IGF-II, MSTN, ghrelin and progastricsin, whereas it had no effect on the FGF6 mRNA levels. Taken together these data may indicate that these genes participate in promoting sea bass compensatory growth induced by refeeding. Future studies are necessary, however, to completely clarify the underlying mechanism of each gene activation in sea bass. Indeed, the present study is the first one to investigate the behavior of the transcripts of those genes over such a long period of fasting and subsequent refeeding.

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7

Diversity and Aquaculture Exploitation of Ictaluridae Species in Mexico

FAUSTINO RODRÍGUEZ ROMERO

7.1 INTRODUCTION

The cultivation of catfish of the Ictaluridae family represents the best success of continental aquaculture in the USA. The first culture was done in the state of Mississippi in the 1960s, but the industry was not really developed until the seventies (Tucker and Robinson, 1990). With the solid support of local banking institutions, universities and the State, the catfish industry has achieved great advances and surprising production figures; from less than 20,000 tons in 1980, to more than 250,000 tons presently (USDA, 2001). Although it is known that in North America and Central America there are around 39 species of *Ictalurus* (Nelson, 1994), the significant catfish piscicola industry that has been developed in the United States and Tamaulipas is based on the culture of *Ictalurus punctatus* Rafinesque, 1818, a native species that has been extensively distributed throughout North America from Canada to northern Mexico. In Mexican territory, there are from 13 to 15 species of catfish that belong to this same genus, that have not been studied to define the closeness of relationship with *I. punctatus*; that could represent opportunities of genetic richness for biotechnological purposes.

7.2 THE PRESENCE OF THE ICTALURIDAE FAMILY IN MEXICO

It is important to characterize the presence of the Ictaluridae family in Mexico for the purpose of establishing the potential of these fish to be exploited through biotechnology aquaculture procedures. The Ictaluridae family is made up of 7 genera and 47 species. In Mexico the little studied Genus *Ictalurus* is represented by between 13 and 15 species, which could be of great interest as genetic reservoirs for the improved use of this resource. The dispersion of these organisms is closely linked to the hydrography. Because of its characteristics of adaptability, resistance and conversion of aliments, which are highly positive for pisciculture, the principal specie exploited for commercial purposes is the *I. punctatus*. Their natural populations seem to be characterized by a wide genetic variability; possibly because of the exchange of genes that the hydrological networks of North America, where they are found in their native habitat, provide. It has been shown that some species of the Genus Ictalurus are capable of hybridizing, both spontaneously and through experimental induction. The most relevant case is the crossing of *I. punctatus* with *I. furcatus*, which offers much better advantages. Other possibilities of hybridization exist in Mexican territories with species like *I. pricei* and *Ictalurus sp.,* Chihuahua catfish or other species of the center and south of the country. Therefore, an evaluation is recommendable for the benefit of Mexican pisciculture and for the conservation of genera, given that these species could offer characteristics attractive to the market and aid in the conservation of the biodiversity and of the genetic richness of this group. The strategies of exploitation for aquaculture in the State of Tamaulipas has been recently focused on the reproduction and fattening of these fish on separate farms, in order to achieve better results in their commercial production.

7.3 PROSPECTIVE STUDIES

Given that the major exploitation of catfish by aquaculture is done in the State of Tamaulipas, fieldwork campaigns were carried out in localities with bodies of water used in the culture of *Ictalurus punctatus* (Figure 7.1) in different parts of the region, to find out the state of exploitation, aquaculture methods, and the results obtained on these farms. The physicochemical parameters of the pH, temperature, salinity, turbidity, alkalinity, ammonium and oxygen dissolved in the aquaculture farms were determined. This data were compared with the information

provided by the aquaculturists (Table 7.1). For the purpose of establishing the diversity of genera and species common in our country and establishing the level of knowledge about their exploitation, a pertinent bibliography was selected and consulted in order to determine the current knowledge of this species in the United States of America and the diversity of species of Ictaluridae in Mexico.

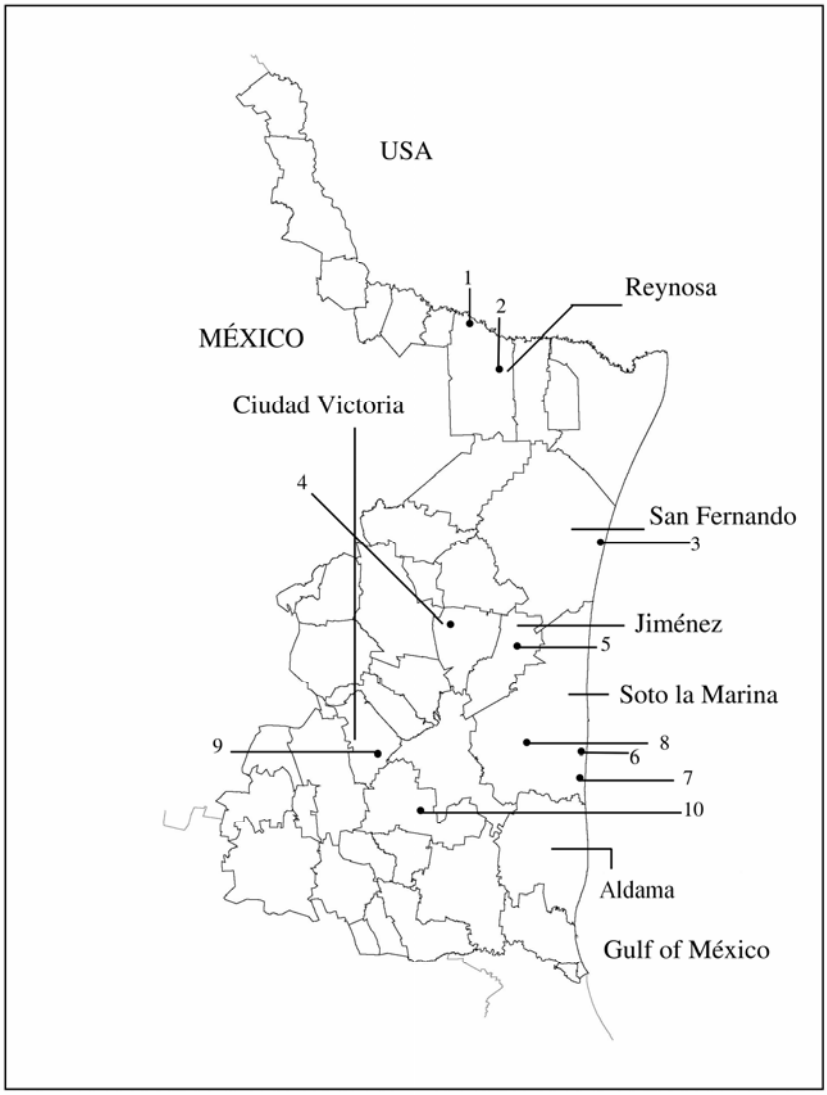

Figure 7.1: Main fish farms of Ictalurus punctatus distribution in the Tamaulipas State, Mexico.

Table 7.1: Ranges of environmental parameter values found in the farms visited

pH	Temperature	Salinity	Secchi	Ammonium
7-7.5	24-31°C	0-6%	15-40 (cm)	Imperceptible

7.4 RESULTS

From the literature revised, it was found that the synonymies and confusion about the taxonomy of some species of catfish were frequent, and although the most accepted opinions are those of Page and Burr (1991) and of Fishbase (2004), in this study a list of 47 species are presented, with those species common to the interior waters of Mexico added, some of them not considered by the above mentioned authors:

Order: Siluriformes
Family: Ictaluridae

Genus:

6. *Ameiurus* (Rafinesque, 1820)
7. *Ictalurus* (Rafinesque, 1820)
8. *Noturus* (Rafinesque, 1818)
9. *Prietella* (Carranza, 1954)
10. *Pylodictis* (Rafinesque, 1819)
11. *Satan*, (Hubbs and Bailey, 1947)
12. *Trogloglanis* (Eigenmann, 1919)

a) *Ameiurus*

i) *Ameiurus brunneus* (Jordan, 1877)
ii) *Ameiurus catus* (Linnaeus, 1758)
iii) *Ameiurus melas* (Rafinesque, 1820)
iv) *Ameiurus natalis* (Lesueur, 1819)
v) *Ameiurus nebulosus* (Lesueur, 1819)
vi) *Ameiurus platycephalus* (Girard, 1859)
vii) *Ameiurus serracanthus* (Yerger and Reylea)

b) *Ictalurus*

i) *Ictalurus australis* (Meek, 1904)
ii) *Ictalurus balsanus* (Jordan and Zinder, 1899)
iii) *Ictalurus dugesii* (Bean, 1880)
iv) *Ictalurus furcatus* (Valenciennes, 1840)

v) *Ictalurus lupus* (Girard, 1858)
vi) *Ictalurus mexicanus* (Meek, 1904)
vii) *Ictalurus ochoterenai* (de Buen, 1946)
viii) *Ictalurus pricei* (Rutter, 1896)
ix) *Ictalurus punctatus* (Rafinesque, 1818)

c) *Noturus*

i) *Noturus albater* (Taylor, 1969)
ii) *Noturus baileyi* (Taylor, 1969)
iii) *Noturus elegans* Taylor, 1969)
iv) *Noturus eleutherus* (Jordan, 1877)
v) *Noturus exilis* (Nelson, 1876)
vi) *Noturus flavater* (Taylor, 1969)
vii) *Noturus flavipinnis* (Taylor, 1969)
viii) *Noturus flavus* (Rafinesque, 1818)
ix) *Noturus funebris* (Gilbert and Swain, 1891)
x) *Noturus furiosus* (Jordan and Meek, 1889)
xi) *Noturus gilberti* (Jordan and Evermann, 1889)
xii) *Noturus gyrinus* (Mitchill, 1817)
xiii) *Noturus hildebrandi* (Bailey and Taylor, 1950)
xiv) *Noturus hildebrandi lautus* (Taylor, 1969)
xv) *Noturus insignis* (Richardson, 1836)
xvi) *Noturus lachneri* (Taylor, 1969)
xvii) *Noturus leptacanthus* (Jordan, 1877)
xviii) *Noturus miurus* (Jordan, 1877)
xix) *Noturus munitus* (Suttkus and Taylor, 1965)
xx) *Noturus nocturnus* (Jordan and Gilbert, 1886)
xxi) *Noturus phaeus* (Taylor, 1969)
xxii) *Noturus placidus* (Taylor, 1969)
xxiii) *Noturus stanauli* (Etnier and Jenkins, 1980)
xxiv) *Noturus stigmosus* (Taylor, 1969)
xxv) *Noturus taylori* (Douglas, 1972)
xxvi) *Noturus trautmani* (Taylor, 1969)

d) *Prietella*

i) *Prietella lundbergi* (Walsh and Gilbert, 1995)
ii) *Prietella phreatophila* (Carranza, 1954)

e) *Pylodictis*

i) *Pylodictis olivaris* (Rafinesque, 1818)

f) *Satan*

i) *Satan eurystomus* (Hubbs and Bailey, 1947)

g) *Trogloglanis*

i) *Trogloglanis pattersoni* (Eigenmann, 1919)

In the case of the Mexican species of catfish, Espinosa Pérez *et al*. (1993) have identified the following species (Figure 7.2):

Figure 7.2: Distribution of catfish species in Mexico: 1. Panuco catfish, 2. Balsas Catfish, 3. Lerma catfish, 4. Blue catfish, 5. Wolf catfish, 6. Ictalurus cf. lupus, 7. Rio Verde Catfish, 8. Yaqui catfish, 9. Channel catfish, 10. Tunal catfish, 11. Cuatro Ciénegas catfish, 12. Mexican Blind catfish, 13. Flathead catfish.

1. *Ictalurus australis* (Meek) – 'Panuco catfish'

Typical location: Forlon, Tamaulipas; Distribution: The south of Veracruz, from Río Blanco to Río Panuco.

2. *Ictalurus balsanus* (Jordan and Snyder) – 'Balsas catfish'

Typical location: Rio Ixtla, Puente de Ixtla, Morelos; Distribution: Endemic in the Río Balsas basin.

3. *Ictalurus dugesi* (Bean)- 'Lerma catfish'

Typical location: Rio Turbio, Guanajuato; Distribution: Endemic in the basins of the Lerma-Santiago, Ameca, Armeria rivers and Chapala Lake.

4. *Ictalurus furcatus* (Le Sueur)- ‘ Blue catfish’

Typical location: New Orleans. Distribution: from the Panuco, Tamesi and Soto La Marina rivers to Iowa and Ohio, USA).

5. *Ictalurus lupus* (Girard)- ‘Wolf catfish’

Typical location: Rio Pecos, USA; Distribution: Ríos Bravo and Soto La Marina.

6. *Ictalurus cf. lupus* (Miller, 1986).

Distribution: Endemic in the Rio Grande; Note: Miller (1986) distinguishes two forms of *I. lupus*; there is no reference to the second form.

7. *Ictalurus mexicanus* (Meek)- ‘Rio Verde catfish'

Typical location: Rascon, San Luis Potosí; Distribution: Endemic in the Rio Panuco.

8. *Ictalurus pricei* (Rutter)- ‘Yaqui catfish’

Typical location: San Bernardino, a tributary of the Rio Yaqui to southern Arizona; Distribution: Endemic in the basins of the Yaqui and Mayo rivers in Sonora, Chihuahua and Durango. They have also been registered in the Casas Grandes and Fuerte rivers.

9. *Ictalurus punctatus* (Rafinesque)- ‘Channel catfish’

Typical location: Ohio River, USA; Distribution: From the Rio Panuco in the south to the Great Lakes region, USA. They have been registered as introductions in other localities of Baja California North, Sonora, Tamaulipas, Jalisco and Nayarit.

10. *Ictalurus* sp. Miller (1986)- ‘Tunal catfish’

Distribution: Basins of the Tunal-Santiaguillo and Nazas-Aguanaval rivers.

11. *Ictalurus* sp. (Williams *et al*., 1989) – ‘Cuatro Cienegas catfish’

Distribution: Cuatro Cienegas, Coahuila.

12. *Prietella phreatophila* (Carranza) – ‘Mexican Blind catfish’

Typical location: Pozo at the foot of the Sierra de Santa Rosa in the Muzquiz municipality, Coahuila; Distribution: Endemic species of the caves of the State of Coahuila.

13. *Pylodictis olivaris* (Rafinesque) – 'Flathead catfish'

Typical location: Ohio River, USA; Distribution: From the north of the Rio Panuco to the south of the United States in the Mississippi Valley.

Information from the CONABIO (2004) indicates the presence of *Ictalurus australis, I. furcatus,* and *I. punctatus*, in the confluence of the Huastecas *(*Veracruz, San Luis Potosi, Hidalgo and Queretaro), while studies carried out by Amezcua-Linares and Yañez-Arancibia (1980) and García-Prieto (1990) report on the presence of *Ictalurus meridionales* and Lamothe (1987) on *I. dugesi.*

7.5 THE CATFISH *ICTALURUS PUNCTATUS* (CHANNEL CATFISH)

Channel catfish can reach a size of 1.2 m. and weigh up to 26.3 kg; have a big and thick head, small eyes and a large mouth. Around the mouth they have two appendixes, similar to whiskers, called 'barbels', with four under the jaw, two above it, and one at each extreme of the jaw. The first dorsal fin and the pectorals have a soft radius, with a modified and strong spine. They have a second adipose fin; the caudal fin is deeply forked. This species of catfish is the only one with spots, having a deeply forked caudal fin and a rounded anal fin. Generally, they have a blue to olive green colored dorso contrasting with a whitish belly; the body is laterally compressed.

7.5.1 Habitat

Freshwater Demersal, pH range: 6.0 - 8.0, depth range 15 m. These are found in the wild state at the bottom of streams with a moderate flow, and in reservoirs and lakes. They are nocturnal, and during the day they stay hidden in pits or holes in rocks or submerged logs. In their natural state, they are found in the eastern part of Central United States and the south of Canada. They are found throughout the Mississippi River to the northeast of Mexico; Also from the eastern St. Lawrence, all along the western rise of the Appalachians to central Florida. They have been widely introduced for the purpose of sport fishing in almost all of the United States.

7.5.2 Reproduction

Ictalurus punctatus is monogamous with an extensive courtship behavior. The male and the female pair up in summer but the relationship is

established well beforehand. Spawning takes place when the male swims alongside the female in the opposite direction. Their tails wrap around their heads and when the body of the male trembles, the female is stimulated and the eggs and sperm are released. The egg mass is deposited in a nest built by the female or both of them. After mating, the male takes charge of the eggs until they hatch (Mayhew, 1987).

7.5.3 Stages of Culture

In culture, the eggs are removed from the spawning tanks and kept in floating trays with water movement until hatching is achieved. Hatch time depends on the temperature, varying between 5 and 10 days after release. The larvae with sac are maintained for 12 to 16 days, a period in which the viteline sac is reabsorbed so they are in condition to receive food. Fattening begins when the fry reach a weight of between 10 and 30 g. earthen ponds of different dimensions that range from a surface of 1 to 10 hectares are used with an average depth of 1.2 m. These ponds are stocked with alevins, at a rate of 8.000 to 12.000 fish per hectare and they ate fed on an artificial diet. At the end of the fattening stage, which could last between 14 and 20 months, fish with an average weight of between 500 and 800 g are harvested (commercial size). Although in the wild this catfish species can reach a weight of nearly 25 kg and a length of 1.2 m, the pisciculturists harvest them when they reach an average size of 2 to 2.5 kg, which is the weight reached after being maintained in captivity during approximately 18 to 24 months. The most common tanks can produce approximately 3 tons of catfish per year.

7.6 EXPLOITATION OF ICTALURIDAE IN MEXICO

The State of Tamaulipas is the major producer of catfish in the country. It has about 95 thousand hectares of surface in limnetic water reservoirs. Their main fishing resources in these bodies of water are represented by 3 species of crayfish, 6 of prawn, 6 of carp, 1 of lobine, 1 of frog, 5 of tilapia and 7 of catfish. Over the last five years, aquaculture in this state has shown an accelerated growth rate. This is a consequence of its natural potential in Tamaulipas, with its excellent development perspective and the strong thrust granted by the state government. In 2001, aquaculture production reached 2,100.76 tons. Catfish represented 53% of production with a volume of 1,117.2 tons, corresponding to the specie *Ictalurus punctatus*. The perspectives of aquaculture in the State indicate a potential of 157 thousand hectares suitable for the culture of

freshwater species (SEMARNAT, 2000). According to official state figures, 67 operating aquaculture farms exist, of which 39 culture *I. punctatus*. The consortium Promotora Acuícola de Tamaulipas formed by the farms: Acuicultores de la Lajilla, Prodatec de Tamaulipas, Granjas Acuícolas Del Noreste, Aquamex and Acuaque, is one of the most important producers in the State with a production in 2003 of about 700 tons. For the culture of this species, spawning tanks and semicircular tubs are used for the development of alevins. The production tanks are rectangular and frequently measure 1 hectare of surface by 1 meter of depth. Some farms, like Aquamex, are dedicated to the development of alevins and juveniles which are brought to other farms for their final growth stage in the south of the State of Tamaulipas. The value ranges of the physicochemical parameters in the bodies of water at the farms visited were found within the average values for the farms in Tamaulipas registered by the aquaculturists in the region. Some of the most important problems in fish production were found, particularly: high rate of blood relationship, genetic quality of the organisms in exploitation is unknown, presence of leeches, nematode parasites in the eyeball, protozoary and aeromona, harmful bacteria and in some cases, fungal infestations.

7.7 *ICTALURUS PUNCTATUS*, AS A BIOTECHNOLOGICAL INTEREST RESOURCE

The huge commercial interest that catfish represent on the American continent has motivated intense exploitation through aquaculture in such a way that presently this resource is cultured throughout the USA, particularly in the Eastern and Central States. In Mexico, the greatest catfish production has been achieved in the State of Tamaulipas, which holds first place in the production of these organisms on a national level with about 2100 tons of *I. punctatus*. However, this value represents just 1% of the production in the United States. Although it is known that in other States they struggle to achieve commercial production of catfish, the only specie exploited is *I. punctatus*, even though in the country there are at least 13 species registered whose potential to be commercially exploited is unknown.

7.8 GENETIC RESOURCES OF ICTALURIDAE SPECIES IN EXPLOITATION

7.8.1 Genetic Variability

The use of microsatellites as genetic markers in catfish of the species *I. punctatus (Liu* and Dunham 1998; Mickett *et al*., 2003) and *I. furcatus* has permitted the determination of high values of genetic polymorphism in these species and their hybrids. The existing high rate of AFLP and RAPD polymorphism between these two species is currently considered a useful tool in the preparation of the genetic map of *I. punctatus* (Liu, 1998). On the subject, Liu *et al.* (2003) published a genetic linkage map, prepared through the use of AFLP where a total of 607 markers were analyzed using 65 combinations of primers in a retrocross of these two species, which will be of great use in commercialization strategies of the hybrids of these species. Geoffrey *et al.* (2001) prepared a linkage map based on the analysis of microsatellites. Recently, Bosworth *et al*. (2004) and Li *et al*. (2004) have evaluated the genetic advantages of the hybrids in relation to the commercial quality of the product. In Mexico there are possibilities of interspecific hybridization in *Ictalurus,* though unknown, but of potential interest given the diversity of the species found in our country in their natural state. Particularly interesting is the possible successful hybridization of *I. punctatus* with *Ictalurus* sp. Chihuahua catfish, *I. pricei*, and possibly with a registered species like *I. meridionalis* (Günther), that could be closely related to *I furcatus* which would be found in Chiapas, widely distributed in the Grijalva and Usumacinta basins. It would be necessary to increase the studies on family relationships and evaluate the genetic resources among the Mexican species related to *I. punctatus,* with the purpose of establishing the possibility of interspecific hybridization and its usefulness in genetic improvement of this genus and its aquaculture exploitation.

7.8.2 Cytogenetics and Cytotaxonomy for Chromosome Manipulation

This specialty currently contributes solid opinion on the family relationships among species of the same genus and constitutes one of the procedures in current use in biotechnology for the exploitation of species by aquaculture. It consists in the genetic manipulation through chromosomatic manipulation, given that through this one can obtain haploid organisms, triploids, gynogenetics, androgenetics, as well as the reversion of sex among others (Rodríguez-Romero and Gasca Montes de

Oca, 1999). These procedures are used in the search for genetic improvement or the profitable exploitation of culturable species. That is why, it is indispensable to know the fundamental karyotypic features of the organisms, such as the number of diploid, fundamental number, morphology of the chromosomes and their classification, and if possible, the characterization of the meiotic karyotype. Even though Channel catfish represent the majority in the aquaculture industry in the United States, many studies are still lacking on cytogenetics and cytotaxonomy in the Ictaluridae family and on the genus *Ictalurus*. More studies would permit the establishment of phylogenetic relationships from the chromosomatic point of view for the purpose of designing chromosomatic manipulation experiments for pisciculture applications. In the studies now published, diploid numbers of 56 have been determined in *I. punctatus* and *I. fucatus*, (LeGrande *et al.*, 1984; Gregory, 2003), *I. melas* (2n=60) and *I. natalis* (2n=62) (Clark and Mathis, 1982). A sex determination model of XX/XY has been accepted in *I. punctatus*, even though chromosome heteromorphism has not been found. No solid evidence at a molecular level that cytogenetically supports this model, even when research was carried out in search of the presence of indicators like the Bkm minisatellite, the telomeric sequence $(TTAGGG)_7$ and the specific genes of the chromosome Y: ZFY and SRY, which are associated to the sex in amniotes (Tiersch et al., 1992). The differentiation in sister chromatides have been found in the majority of chromosomes at 83-100% in presence of BrdU over two cellular cycles. The exchange of sister chromatides has been observed from between 1 to 4% of the chromosomes, a value that stays within the exchange values of chromatides presented spontaneously in the chromosomes of eukaryotes.

7.8.3 Cytogenetics of Hybrids

I. punctatus and *I. furcatus* have 29 pairs of chromosomes (Wolters *et al.*, 1981; LeGrande *et al.*, 1984; Zhang and Tiersch, 1997) with a genome size of ~1 x 10^9 pb (Tiersch *et al.*, 1990; Tiersch and Goudie, 1993). The experimental hybridization work done by LeGrande *et al.* (1984), have shown that resulting karyotype for the hybrids of these two species are stable and similar in diploid number, fundamental number and chromosomatic morphology, to progenitor species, which allows for the deduction of a close family relationship between these two species and an ample possibility to apply the advantages of the hybrid to favor greater and improved productivity in aquaculture. From the Cytotaxonomy point of view, there is a lack of information regarding the structure of

karyotypes in most Ictaluridae species; the structure of karyotypes is practically unknown in the species of Mexico.

7.9 GENETIC IMPROVEMENT PERSPECTIVES IN *ICTALURUS PUNCTATUS*

The lack of genetic selection sustained in *I. punctatus* has effectively maintained a high level of phenotypical variation in commercial and wild populations. The pisciculturists also have access to the wild populations in order to increase genetic variation of the exploited populations, if need be. The numerous descendents, together with the heritability estimations for some features (Tave, 1986), promise greater genetic gains and better expectations in the increase of positive qualities in this species in aquaculture.

7.10 THE BIODIVERSITY OF ICTALURIDAE IN MEXICO AND THEIR POTENTIAL AS A GENETIC RESOURCE FOR AQUACULTURE BIOTECHNOLOGY

The thirteen to fifteen species found in the waters within Mexico represent a potential genetic resource that should be studied for their possible use in genetic improvement and exploitation of cultivated species, such as *I. punctatus* of Tamaulipas, and wild populations of the species, that have been registered in our country. From the scientific knowledge point of view of these resources, there is much research to be done. It would allow for firm taxonomic identification of these species and establish the family relationships on the species and genus levels. On the matter, the only antecedent is that which Perez-Ponce de León and Choudhury (2002) contributed in a study of empiric evidence of the biogeographic patterns of *Ictalurus* in Mexico. Special attention should be paid to possibly identified species such as *Ictalurus australis* 'Panuco catfish', *Ictalurus balsanus* 'Balsas catfish', *Ictalurus dugesi* 'Lerma catfish', *Ictalurus mexicanus* 'Rio Verde catfish', *Ictalurus pricei* (Rutter)- 'Yaqui catfish', *Ictalurus* sp. 'Tunal catfish', *Ictalurus* sp. 'Cuatro Cienegas catfish', *Ictalurus cf. lupus (*Miller, 1986) and *Ictalurus* sp. 'Chihuahua catfish', as these may be taxonomic entities closely related to *I. punctatus* (Wolters, 2004). Also, there are other species in the central and southern parts of the country identified as *I. meridionalis* and *I. dugesi* of which very little is known.

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8

Repetitive DNA Elements as Probes for the Genomic Analysis of Scamp and Yellowmouth Grouper from the Gulf of Mexico

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8.1 INTRODUCTION

The serranids (family, Serranidae) include groupers, scamps, sea basses and basslets among its members (Bullock and Smith, 1991). The western Atlantic fish, scamp (*Mycteroperca phenax*) and yellowmouth grouper (*Mycteroperca interstitialis*), found in tropical and subtropical waters, are important for both sports and commercial fisheries in the Gulf of Mexico. Both species are known to co-exist in the northern, eastern and western gulf regions (Bullock and Smith, 1991) and are difficult to distinguish from each other based on simple morphological criteria (Smith, 1971). Identification between scamp and yellowmouth groupers is further complicated by their similar meristic characteristics (Smith, 1971).

Taxonomy and identification of fish has been based on morphology and modern molecular tools of DNA and protein analysis (Marr, 1957; Beverstock *et al*., 1979; Avise *et al*., 1986; Bermingham and Lamb, 1986; Turner *et al*., 1991; Wirgin *et al*., 1991; Boisneau *et al*., 1992; Dahle, 1994; Wirgin and Waldman, 1994). More recently, PCR (polymerase chain reaction) amplification of random amplified polymorphic DNA fragments using either arbitrary primers or primers for highly-repeated DNA elements has been quite useful in fish genomic analysis and species identification (Dinesh *et al*., 1993; Perring *et al*., 1993; Callejas and

Ochando, 1998; Qiu and Li, 1999; Yoon and Kim, 2001; Ali *et al*., 2004; Ahmed *et al*., 2004; Ali *et al*., 2004; El-Zaeem and Ahmed, 2006). It is possible, therefore, that a similar approach of RAPD analysis may aid in resolving the confusion between these two fish, scamp and yellowmouth grouper.

Telomeres constitute the terminal segments of eukaryotic chromosomes (Blackburn, 1994; Abuin *et al*., 1996; Baird, 2006). These elements are important in maintaining chromosome integrity, stability and synapsis (Zakian, 1989; Bhattacharyya and Lustig, 2006; Figueiredo *et al*., 2002; Stewart and Weinberg, 2006). Additional data suggest importance of telomere integrity and maintenance in cellular aging and immortality (Zakian, 1989; Kipling and Cooke, 1992; Baird, 2006). Sequence analysis has revealed eukaryotic telomere to consist of (TG) repeat (Blackburn, 1994). The repeat nature of telomere has been conserved through evolution since similar sequence(s) are present in most other eukaryotic telomeres including human. *In situ* hybridization analysis has also revealed presence of $(TTAGGG)_n$ in many vertebrates (Meyne *et al*., 1989). A high level of telomerase activity has been also noted in fish by Klapper *et al*. (1998). In a study on sex determination in channel catfish (*Ictalurus punctatus*) and four salmonid species, the presence of telomeric sequence $(TTAGGG)_n$ was also documented by Southern analysis with a human telomeric probe (Tiersch *et al*., 1992; Abuin *et al*., 1996). However, information about the presence of telomeric sequences and size in amberjack, scamp and yellowmouth grouper has been lacking.

In the present study, therefore, arbitrary primers as well as primers for the repeated DNA elements were used for PCR amplification of both scamp and yellowmouth grouper DNA. We also sought to determine the genomic variability, if any, of scamp from three different regions of the Gulf of Mexico. The experiments described here demonstrate that unique, reproducible, bands (and banding patterns) can be obtained using primers for repeated-DNA elements in these fish populations. Additionally, we report the terminal restriction fragment (TRF) size analysis and variation in these two closely related fish species.

8.2 MATERIALS AND METHODS

8.2.1 Tissue Sample and DNA Isolation

Liver samples were collected from fish at three different locations, mid-eastern, north-central and north-western, from the Gulf of Mexico: yellowmouth grouper (*Mycteroperca interstitialis*) from St. Petersburg, FL (mid-eastern region); scamp (*M. phenax*) from St. Petersburg (SP; mid-eastern region), Destin, FL (DT; north-central region) and Chauvin, LA (LA; north-western region); and greater amberjack (*Seriola dumerili*) from Destin, FL (AM; north-central region). The liver tissue was excised from the intact fish on the docks with a sterilized scalpel and stored on dry ice. The samples were stored in a -70°C freezer for long-term storage, if needed.

For DNA extraction, approximately 1g of tissue was removed from each sample, placed on dry ice and pulverized to a powder using a pestle and a mortar. The pulverized tissue samples were extracted initially using a CTAB (cetyltrimethyl ammonium bromide) method (Rogers and Bendich, 1988). DNA obtained from the CTAB method was further purified using the standard SDS/phenol/chloroform method (Davis and Dibner, 1986). Before re-extraction, the samples were treated with DNase free-RNase (1.5 μg/μl) to remove any contaminating RNA. Following phenol/chloroform extraction, the DNA was precipitated with 2 volumes of cold 100% ethanol. The precipitated DNA was recovered, washed with cold 70% ethanol and dried under vacuum. All samples were stored in 50 μl of TE at 4°C until further use (Davis and Dibner, 1986).

8.2.2 PCR Analysis

Twenty-six primers (5-mer, 7-mer and 10-mer primers) were selected for use in PCR studies. Initially, PCR was performed following the manufacturer's recommendations. All reagents were provided in the Gene Amp PCR Reagent Kit (Perkin Elmer, Norwalk, CT). The standard procedure used for PCR amplification in the Thermal Cycler involved initial heating of the samples to 94°C for 5 minutes, followed by 30 cycles of (i) heating to 94°C (1 min) for the denaturation step, (ii) cooling to a predetermined temperature for the annealing step (0.5 min) and, (iii) primer extension at 72°C (1.5 min). After 30 cycles of amplification, the samples were reheated to 94°C for 2 min then left at 4°C until removed. The optimal annealing temperatures used in this study were experimentally determined to be 62°C for the 15-mer primers and 50°C for the 16-mer and 10-mer (random) primers.

8.2.3 Restriction Enzyme Digestion, Gel Electrophoresis and Southern Analysis

The restriction enzyme digestion of the purified DNA and 5' DNA terminus labeling of the 24-mer $(TTAGGG)_4$ oligonucleotide with γ-^{32}P-ATP were carried out following manufacturer's (Promega) recommendations.

Gel electrophoresis analysis of PCR products was carried out using composite agarose/acrylamide gels. The composite gels consisted of 3% acrylmide and 0.5% agarose. Briefly, 0.5% agarose solution (in H_2O) was brought to a boil and then combined with warm 3% acrylamide solution (acrylamide, 5X TBE, 10 μL TEMED) in a 50 ml Erlenmeyer flask. Immediately, a 200 μL aliquot of ammonium persulfate solution (100 mg/ml) was added and mixed. The gel solution was poured into the glass plate assembly. Polymerization of the gel was allowed to proceed for 1 hour at room temperature. The gel was pre-run at 25 volts for 5 minutes in 1X TBE as running buffer. Samples were added and electrophoresced for 1.5 hrs at 45 volts in a vertical slab gel apparatus (Hoefer Instruments, San Francisco, CA). Following electrophoresis, the gel was carefully removed and stained with ethidium bromide (1 μL/ml). The DNA fragments were visualized using a 320 nm UV Transilluminator (Spectroline, Westbury, NY) and photographed using a Polaroid camera.

For TRF analysis, restriction digests were analyzed by electrophoresis in 0.9% agarose gels and stained with ethidium bromide for visualization and photography. Southern blots were prepared using standard methods as described by Bhatnagar *et al*. (1995). Prehybridization and hybridization of the Southern blots was carried out using the standard procedures except that *Escherichia coli* DNA was used instead of the salmon testis DNA in the prehybridization solution (Bhatnagar *et al*., 1995). Pre-hybridization and hybridization were carried out at 27°C in the presence of 50% formamide in a constant temperature water bath shaker. All blots were washed in 2X SSC twice for 15 minutes each at 25°C, and in 2X SSC twice for 30 minutes at 27°C prior to autoradiography. Only hybrids with > 97% sequence similarity were stable under these experimental conditions. The washed blots were exposed for 3 to 5 days at -70°C for autoradiography (Bhatnagar *et al*., 1995). Statistical analysis was performed using the Microsoft Excel Statistical software package.

8.3 RESULTS

8.3.1 DNA Isolation

DNA extraction from fish liver was accomplished in a single day with the combination of the CTAB and the SDS/phenol/chloroform method. This modified method was more consistent and successful than other published methods for the purification of genomic DNA from frozen fish liver (Davis and Dibner, 1986; Rogers and Bendich, 1988; Taggart *et al*., 1992). Fresh tissues are recommended for most procedures, but the modified CTAB/phenol method is also useful for tissues collected and stored on ice for up to 24 hours. Thus far the method has been successfully used to extract genomic DNA from skeletal muscle, gonad, heart and liver tissue of several species of fish (data not shown). High molecular weight DNA has been extracted from members of various fish families: Serranidae; sea bass (*Centropristis philadelphica*), scamp (*M. phenax*), yellowmouth grouper (*M. interstitialis*), gag (*M. microlepis*), and red grouper (*Epinephelus morio*); Carangidae; greater amberjack (*Seriola dumerili*) and horse-eye jack (*Caranx latus*); Sparidae; pinfish (*Lagodon rhomboides*); Pomacanthidae; asfur angelfish (*Arusetta asfur*); Scombridae; spanish mackeral (*Scomberomorus maculatus*); and Balistidae; grey triggerfish (*Balistes capriscus*) using the method described here (data not shown). DNA extracted from several of these fish species has been used in our laboratory for PCR amplification and TRF analysis (data not shown).

8.3.2 PCR Studies

Of the 26 primers tested in this study (Table 8.1), three sets of primers yielded DNA fragments characteristic of different fish (scamp, yellowmouth grouper and amberjack) upon electrophoresis in composite agarose/acrylamide gels. These primers were $(CAG)_5$, $(ACTG)_4$ and the combination primer $(CAC)_5/(ACTG)_4$. The other primers did not yield DNA fragments useful for discriminating these fish samples from each other or yielded banding patterns unsuitable for analysis. Although a number of bands were seen with $(CAC)_5$, $(ACTG)_4$ and the combination primer $(CAC)_5/(ACTG)_4$ but the most informative bands were <2 Kb in size. Therefore, the following analysis is based on a systematic examination of these bands in the composite agarose/acrylamide gels.

Table 8.1: List of primers tested for PCR amplification of yellowmouth grouper, scamp and amberjack DNA

Primer No.	Primer Sequence
1	CAGCAGCAGCAGCAG
2	CACCACCACCACCAC
3	GTGGTGGTGGTGGTG
4	TCCTCCTCCTCCTCC
5	ACTGACTGACTGACTG
6	GACAGACAGACAGACA
7	TTAGGGTTAGGGTTAGGGTTAGGG
8	GTGCAATGAG
9	CAATGCGTCT
10	AGGATACGTG
11	TCCCTTTAGC
12	CGAATAACTG
13	AGGTTCTAGC
14	TCCGACGTAT
15	GGAAGACAAC
16	AGAAGCGATG
17	GCATTAACGC
18	CAGCAGCAGCAGCAG/GACAGACAGACAGACA
19	CACCACCACCACCAC/ACTGACTGACTGACTG
20	CACCACCACCACCAC/TTAGGGTTAGGGTTAGGGTTAGGG
21	CAGCAGCAGCAGCAG/TTAGGGTTAGGGTTAGGGTTAGGG
22	CAGCAGCAGCAGCAG/ACTGACTGACTGACTG
23	CAGCAGCAGCAGCAG/GTGCAATGAG
24	CACCACCACCACCAC/CAGCAGCAGCAGCAG
25	TTAGGGTTAGGGTTAGGGTTAGGG/TCCGACGTAT
26	ACTGACTGACTGACTG/AGGTTCTAGC

8.3.2.1 PCR Analysis of $(CAG)_5$ Primer

Electrophoretic analysis of the PCR amplified-$(CAG)_5$ primed DNA samples showed unique species-specific banding patterns (Figure 8.1; Table 8.2). The amberjack samples possessed three fragments that are <2 Kb in size, of which two are shared with both scamp and yellowmouth grouper (1425 and 1050 bp). Amberjack possessed a unique band at 1750 bp (Figure 8.1; Table 8.2). Also, amberjack samples lacked bands of 1700, 1200, 1125, 940, 920 and 835 bp that were present in all samples of scamp and the yellowmouth grouper. DNA

fragments of 1125, 940 and 920 bp was unique to yellowmouth grouper for this primer and gave a unique ladder like appearance. The scamp from each sample site generally shared bands but lacked the bands specific to yellowmouth grouper and amberjack (Figure 8.1; Table 8.2). A 750 bp fragment was present in scamp from two locations (DT and LA) but was not amplified in samples from the mid-eastern Gulf of Mexico (SP).

Figure 8.1: Composite agarose-acrylamide gel analysis of PCR products. $(CAG)_5$ primer was used in this experiment using genomic DNA from amberjack (AM), yellowmouth grouper (IS) and scamps from St. Petersburg, Florida (SP), Destin, Florida (DT) and Chauvin, Louisiana (LA), respectively.

Table 8.2: PCR analysis with $(CAG)_5$ primer*

DNA Fragment (bp)	Amberjack	YM Grouper	Scamp (SP)	Scamp (DT)	Scamp (LA)
1750	+				
1700		+	+	+	+
1425	+	+	+	+	+
1200		+	+	+	+
1125		+		[+]	+
1050	+	+	[+]	+	+
940		+			
920		+			
835		+	+	+	+
750	[+]			+	+

*Size of the amplified band in the composite agarose-acrylamide gel. A "+" sign indicates the presence of a particular band in the gel, whereas, a blank space denotes the absence of the corresponding band in the gel. Symbol within [ ] indicates infrequent amplification in different DNA samples from a particular species. Five or more DNA samples were amplified for each species. Abbreviations: YM, yellowmouth grouper; SP, scamp from St. Petersburg, Florida; DT, scamp from Destin, Florida; LA, scamp from Chauvin, Louisiana.

8.3.2.2 PCR Analysis of $(ACTG)_4$ Primer

Electrophoretic data for the $(ACTG)_4$ primer is summarized in Figure 8.2 and Table 8.3. DNA fragments of 1375, 1225, 1150 and 650 bp were part of the banding pattern unique to amberjack samples. A band at 1900 bp was amplified from both yellowmouth grouper and scamp DNA samples. The 1900 bp fragment was the only fragment amplified for yellowmouth

grouper using this primer. In contrast, scamp samples from all three locations amplified additional bands of 1300 and 1225 bp (Figure 8.2; Table 8.3).

Figure 8.2: Composite agarose-acrylamide gel analysis of PCR products. $(ACTG)_4$ primer was used in this experiment using genomic DNA from amberjack (AM), yellowmouth grouper (IS) and scamps from St. Petersburg, Florida (SP), Destin, Florida (DT) and Chauvin, Louisiana (LA), respectively.

Table 8.3: PCR analysis with $(ACTG)_5$ primer*

DNA Fragment (bp)	Amberjack	YM Grouper	Scamp (SP)	Scamp (DT)	Scamp (LA)
1900		+	+	+	+
1375	+				
1300			+	+	+
1225	+		+	+	+
1150	+				
750	+				

*Size of the amplified band in the composite agarose-acrylamide gel. A "+" sign indicates the presence of a particular band in the gel, whereas, a blank space denotes the absence of the corresponding band in the gel. Five or more DNA samples were amplified for each species. Abbreviations: YM, yellowmouth grouper; SP, scamp from St. Petersburg, Florida; DT, scamp from Destin, Florida; LA, scamp from Chauvin, Louisiana.

8.3.2.3 PCR Analysis of $(CAC)_5/(ACTG)_4$ Primer

Combining primers allowed different nucleic acid sequences to be amplified (Figure 8.3; Table 8.4). Unique banding patterns were observed for amberjack, yellowmouth grouper and scamp DNA samples using the $(CAC)_5/(ACTG)_4$ primer combination. Among the five different fragments (1600, 1160, 890, 790 and 665 bp) that were amplified from the amberjack DNA, only the 890 bp fragment was amplified in yellowmouth grouper and scamp samples (Figure 8.3; Table 8.4). The combination primer amplified DNA fragments of 890, 820, 560 and 330 bp in both yellowmouth grouper and scamp samples. A 1600 bp fragment was only amplified from the scamp DNA and was lacking in the yellowmouth grouper DNA (Figure 8.3; Table 8.4).

Figure 8.3: Composite agarose-acrylamide gel analysis of PCR products. $(CAC)_5/(ACTG)_4$ primer combination was used in this experiment using genomic DNA from amberjack (AM), yellowmouth grouper (IS) and scamps from St. Petersburg, Florida (SP), Destin, Florida (DT) and Chauvin, Louisiana (LA).

Table 8.4: PCR analysis with $(CAC)_5/(ACTG)_5$ primer*

DNA Fragment (bp)	Amberjack	YM Groupe	Scamp (SP)	Scamp (DT)	Scamp (LA)
1600	+		+	+	+
1260					+
1160	+				
1000			[+]		
890	+	+	+	+	+
820		+	+	+	+
790	+				
665	+				
560		+	+	+	+
330		+	+	+	+

*Size of the amplified band in the composite agarose-acrylamide gel. A "+" sign indicates the presence of a particular band in the gel, whereas, a blank space denotes the absence of the corresponding band in the gel. Symbol within [ ] indicates infrequent amplification in different DNA samples from a particular species. Five or more DNA samples were amplified for each species. Abbreviations: YM, yellowmouth grouper; SP, scamp from St. Petersburg, Florida; DT, scamp from Destin, Florida; LA, scamp from Chauvin, Louisiana.

8.3.3 TRF Analysis

TRF size analyses for each sample group of carrangid (amberjack, AM) and serranid fishes [yellowmouth grouper, IS; and scamp from Destin, Fl (DT), St. Petersburg, Fl (SP), and Louisiana, (LA)] were performed following *Hinf*I digestion (Figure 8.4). The TRF of amberjack (1.95 kb) is shorter than the TRF of yellowmouth grouper and scamp from all but one location (Table 8.5). Among scamps, *Hinf*I TRF fragment sizes were significantly smaller in those from Louisiana (2.0 kb) than from Florida (3.15 and 3.16 kb; Table 8.5). Additional TRF size analysis for different fish samples was performed following *EcoR*I, *Hind*III and *Msp*I digestion. No difference in TRF size was observed between amberjack, yellowmouth grouper and scamp in *EcoR*I or *Hind*III digests (data not shown). However, the average peak TRF value for each fish sample digested in *Msp*I indicated amberjack TRF (~1.7 kb) to be much smaller than the TRF of any of the serranids (3.3-3.7 kb; data not shown). Interestingly,

yellowmouth grouper TRF peak value (3.1 kb) was found to be smaller than the scamp TRF peak value (3.3-3.7 kb) for DNA digested with *Msp*I (data not shown).

Figure 8.4: Southern analysis of TRFs in genomic DNA digested with HinfI from amberjack (AM), yellowmouth grouper (IS) and scamp from St. Petersburg, FL (SP), Destin, FL (DT) and Chauvin, Louisiana (LA), respectively. For electrophoretic analysis, approximately 5 μg of HinfI-digested DNA was loaded in each lane of a 0.9% agarose gel and subjected to electrophoresis at 45 volts for 1.5 hours in 1X TAE (0.04 M Tris-acetate, 0.002 M EDTA, pH 8.0) as a running buffer. A 2 kb ladder of DNA (BRL, Gaithersburg, MD) was used as a marker (left column). Following electrophoresis, the gel was stained with ethidium bromide (1 μg/ml), photographed and Southern blotted onto a nylon membrane for autoradiography. Fish TRFs were identified using a ^{32}P-labelled $(TTAGGG)_4$, a human telomeric DNA sequence.

Table 8.5: Average TRF size (Kb)*: Hinf1 digest

Species	Peak (+STD)	Mean (+STD)
Amberjack	2.35 + 0.05	1.95 + 0.1000
Grouper	3.16 + 0.05	3.08 + 0.0236
Scamp (SP)	3.23 + 0.09	3.16 + 0.0236
Scamp (DT)	3.36 + 0.12	3.15 + 0.1080
Scamp (LA)	3.23 + 0.19	2.0 + 0.4242

* Average TRF size in Kbp was determined from scans of autoradiograph. The TRF DNA size was estimated at both peak value and the total range of fragments in the autoradiograph. Standard deviations were obtained following standard statistical analysis.

8.4 DISCUSSION

The studies described here demonstrate that the PCR amplification of yellowmouth grouper, scamp and amberjack DNA using $(CAG)_5$, $(ACTG)_4$ and $(CAC)_5/(ACTG)_4$ primers yields unique, reproducible, banding patterns for each fish. Additionally, these studies provide data in support of the use of random primers in identification and characterization of various fish species (Dinesh *et al*., 1993; Ali *et al*., 2004).

Yellowmouth grouper amplified with $(CAG)_5$ primer yielded fragments not found in scamp while the overall DNA ladder-like pattern was missing from all scamp samples. All scamp samples, regardless of the location, yielded DNA fragments of 1300 and 1225 bp for $(ACTG)_4$ primed DNA that were lacking in the yellowmouth grouper DNA.

Notably, a somewhat similar DNA fragment pattern was observed for both scamp and groupers except for the 1600 bp fragment that was present only in scamp DNA. The outgroup, amberjack, DNA yielded patterns uniquely different from scamp and yellowmouth grouper when amplified with the three primer sets. Four of the five amplified fragments in amberjack DNA with the $(CAC)_5/(ACTG)_4$ primer were unique to this fish. This seems to reflect the genetic distance of carangid from the serranid fishes. Although limited, our data also provides evidence in support of local genetic variation (probably due to reproductive isolation) of scamp present in the three different regions (mid-eastern, north-central and north-western regions) of the Gulf of Mexico.

Our studies have documented three new primer sets and their utility in the PCR-based identification method for the discrimination of carangid and serranid fishes. Moreover, these data also permit unambiguous identification of scamp from the yellowmouth grouper. A more detailed study based on a larger sample size may provide further support to the approach outlined in this study.

Additional studies demonstrated the presence of $(TTAGGG)_4$ repeat arrays (TRF) in the genome of *M. interstitialis*, *M. phenax* and *S. dumerili*. These data support and extend similar observations for other eukaryotes that the telomeric sequence is conserved in different vertebrates (Meyne *et al*., 1989; Tiersch *et al*., 1992; Bhatnagar *et al*., 1995; Abuin *et al*., 1996). Mammalian TRF size analysis suggests that TRF size may vary in different species as shown for rat, mouse and higher primate (Bhatnagar *et al*., 1995, Kipling and Cooke, 1990; Hastie *et al*., 1990; Makarov *et al*., 1993). Although the apparent variation in TRF size in *Msp*I and *Hinf*I digests of fish DNA provides some support for TRF size differences in different species, its full potential for interpretation of fish taxonomy needs further study.

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Serological Methods for the Detection of Pathogenic Bacteria in Aquaculture: Present Status and Future Prospects

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9.1 INTRODUCTION

Aquaculture industry has considerably developed during the last decades and is nowadays one of the fastest growing food production sector in the world (Naylor, 2005). Currently, commercial aquaculture represents nearly 41% of the total fishing catch worlwide (FAO, 2003), and indications are that aquaculture will continue to make increasing contributions to fish supplies over the next few years, helping to reduce the gap between supply and demand for fish products. Today, production of farmed salmonid species exceeds wild salmonid fishing captures by about 70% (FAO 2003). Although most aquaculture production to date has been in freshwater species, marine aquaculture has been growing notably. Marine fish culture is dominated by Atlantic salmon (*Salmo salar*) led by Norway, Chile, United Kingdom, Canada and Ireland (FIGIS, 2006). Other economically important marine fish species are gilthead seabream (*Sparus aurata*), European seabass (*Dicentrarchus labrax*), eel (*Anguilla anguilla*) and turbot (*Scophthalmus maximus*), which are reared in Mediterranean (Greece, Italy, France) and southern Atlantic (Spain and Portugal) coasts and yellowtail (*Seriola quinqueradiata*), ayu (*Plecoglossus altivelis*), flounder (*Paralychthys olivaceous*), and seabream (*Pagrus major*) in Japan. Other high-value flatfish, sole (*Solea*

senegalensis), naturally distributed in Mediterranean and Atlantic waters is also reared successfully in extensive aquacultural productions in Spain and Portugal. New species farmed in marine net pens include Atlantic cod (*Gadus morhua*), Atlantic halibut *(Hippoglossus hippoglossus*) and bluefin tuna (*Thunnus* spp.) (Naylor, 2005). Numerous other carnivorous finfish species such as barramundi (*Lates calcarifer*), yellowtail kingfish (*Seriola lalandi*) and southern hake (*Merluccius australis*), are also experimentally or comercially farmed (Weber, 2003). As occurs in other areas of animal production, infectious diseases are an ever-present hazard in aquaculture, with the potential to cause heavy stock losses or to reduce the commercial value of the fish as food for humans. The type, incidence and severity of these diseases may vary according to the raised fish species and the method used for culture. Environmental factors and poor water quality, sometimes associated with self-pollution due to effluent discharge and pathogen transfer via movements of aquatic organisms, appear to be an important cause of mortalities in aquaculture. Moreover, diversification of aquaculture species adds new potential opportunities for fish pathogens and new health concern. Unfortunately, while knowledge of the physiological and nutritional requirements of the cultured species is gradually achieved, the preservation of health status of the new and old species maintained in captivity remains a high priority.

Reliable methods for detection/ identificaton and epidemiological typing of pathogenic organisms in diseased and carrier fish or in other reservoirs in aquatic environment is crucial for the establishment of effective control measures in order to eliminate or reduce the impact of the diseases in farmed fish. The diagnosis of bacterial fish diseases is usually carried out by agar cultivation and identification of the microorganisms on the basis of its phenotypical characteristics and complemented by the use of serological and molecular methods. Many automated identification systems, originally developed for the identification of human pathogens [API 20E, API ZYM, API 20NE, API 50 CH, API Rapid ID 32 (bioMerieux, Marcy-l'Etoile, France), Biolog MicroPlates GN2, GP2, AN (Biolog, Inc., Hayward, CA, USA), Minitek (Becton-Dickinson & Company)], have been applied for the identification of bacterial pathogens isolated from fish and homeothermic animals with different success due to the lack of information about veterinary pathogens in the database or to the existence of false positive and negative reactions for several characteristics (Toranzo *et al*., 1986; Biosca *et al*., 1993; Santos *et al*., 1993; Austin *et al*., 1997; McCasland and True, 2001; Oraic *et al*., 2002, Romalde and Toranzo, 2002; Austin *et al*., 2003; Israil *et al*., 2003; Buller, 2004; Padros *et al*., 2006). Despite recent

improvements, these systems are still of limited reliability for veterinarian and fish pathologists. Moreover, this classical bacteriological method is appropriate for the detection of easily cultured bacteria, but, identification of many pathogens is delayed or impeded by rather fastidious growth and weak reactivity in biochemical test. Alternatively, non-culture-based microbial detection techniques, founded in immunological or genetic principles, have been or are being developed for identification of fish pathogens (Bernoth, 1999; Cunningham, 2002; Osorio and Toranzo, 2002; Adams, 2004; Colorni, 2004; Cunningham, 2004; Adams and Thompson, 2006). These methods have considerable potential for diagnose of disease problems as well as for ecological and epidemiological study of many fish pathogens of interest in aquaculture if they can meet the stringent criteria of a standardized, validated, reliable and accessible diagnostic technique (Hiney, 1997; Hiney and Smith, 1999; Hiney, 2000; Cunningham, 2002, 2004; Adams and Thompson, 2006). This chapter analyses the serological methods that have been developed and are currently in use for diagnosis of main bacterial diseases affecting cultured fish. Although the range of bacterial fish pathogens is steadily increasing, only a relatively small number are responsible of important economic losses in fish held in freshwater and saltwater worldwide (Austin and Austin, 1999; Buller, 2004; Toranzo *et al*., 2005). Table 9.1 serves as a quick reference guide to the diseases reviewed in this chapter.

Table 9.1: Main bacterial diseases affecting cultured fish species worldwide

Disease	Aetiological agent	Cultured fish species
Vibriosis	*Listonella anguillarum (Vibrio anguillarum)*	Salmonids, turbot, seabass, striped bass, eel,ayu, cod, seabream
	L- ordalii (V. ordalii)	Salmonids
	V. salmonicida	Atlantic salmon, cod
	V. vulnificus	Eels
	V. harveyi	Sole
Winter ulcer	*Moritella viscosa (V. viscosus)*	Atlantic salmon
Photobacteriosis	Photobacterium damselae subsp. piscicida (*Pasteurella piscicida*)	Seabass, striped bass, *seabream, yellowtail, sole*
Furunculosis	*A. salmonicida*	Salmonids, turbot
Pseudomonadiasis	*Pseudomonas anguilliseptica*	Seabream, turbot, ayu, eel
Tenacibaculosis	*Tenacibaculum maritimum*	Salmonids, turbot, sole,

	(Flexibacter maritimus)	Seabass, seabream,flounder
	T. gallaicum	Turbot, sole
	T. discolor	Turbot sole
	T. soleae	Sole, sea bass
Streptococcosis	*Streptococcus iniae*	yellowtail, flounder, sea bass, barramundi
	Streptococcus parauberis	Turbot
Lactococcosis	*Lactococcus garvieae*	Yellowtail, eel, salmonids
BKD	*Renibacterium salmoninarum*	Salmonids
Micobacteriosis	*Mycobacterium marinum*	Sea bass, turbot, Atlantic salmon
Piscirickettsiosis	*Piscirickettsia salmonis*	Salmonids
Haemorrhagic septicaemia	*Aeomonas hydrophila*	mainly freshwater fish species
Rainbow Trout Fry síndrome	*Flavobacterium psychrophilum*	Salmonids, eel, carp,tench, ayu
Columnaris disease	*Flavobacterium columnare*	Salmonids and other freshwater fish species
Edwardsiellosis	*Edwardsiella tarda*	various freshwater fish species, turbot
Enteric red mouth	*Yersinia ruckeri*	Salmonids

9.2 IMMUNOCHEMICAL TECHNIQUES FOR DIAGNOSIS OF BACTERIAL FISH DISEASES

The importance of antibodies and the immune response in health and the clinical value of immunological reagents was appreciated long ago. Imunochemistry offers simple, rapid, sensitive and easily automated methods to confirm disease diagnosis, to detect pathogens that cannot be detected by traditional microbiological or by molecular techniques and to detect antibodies to specific pathogens in the serum of animals as an indicator of previous exposure to the microorganism or of infection (Adams, 1992; Adams and Marin de Mateo, 1994; Adams *et al*., 1995; Coll *et al*., 1995; Austin, 1998; Adams, 1999; Sheng *et al.*, 2003; Adams, 2004). However, the sensitivity of such methods for the detection of pathogens in environmental samples and sub-clinical disease is still limited (Adams, 1999; Toze, 1999; Vatsos *et al*., 2002; Madetoja and Wiklund, 2002; Osorio and Toranzo, 2002; Bej, 2003; Adams, 2004).

All immunological techniques are based on a highly specific reaction between an antigen and an antibody. The region of the antigen surface that interacts with the antibody-binding site is the epitope. The antigen-

antibody binding reaction is dependent on reversible non-covalent interactions. The specificity and sensitivity of the binding reaction are determined by the type of antibody (polyclonal or monoclonal). Conventionally, polyclonal antibodies raised in rabbits are used in disease diagnosis, serotyping and other biological applications. Polyclonal antibodies may be produced against a single antigen (monospecific antiserum) or against a mixture of antigens (polyspecific or multispecific antiserum). Although most immunochemical reactions will occur with unfractionated polyclonal antiserum, the use of affinity-purified immunoglobulin fraction reduces the possibility of non-specific interactions or unwanted background reactions. Some of the other limitations of polyclonal antisera such as product standardization, cross reaction, limited quantity, and inability to discriminate antigen at epitope level, could be overcome by the use of monoclonal antibodies (Mab) produced by a well-described hybridoma-technique (Kohler and Milstein, 1975), MAbs have been applied in different areas of biology and biotechnology and are widely used in the diagnosis, serotyping and analysis of antigens in microbial pathogens in medical and veterinary sciences (Bogdnanovik and Langlands, 2001; Adams, 2004). Mabs are also being applied to the study of fish immunology, disease research and diagnosis of fish infectious diseases mainly in research centres and Univerities or reference laboratories (Adams *et al.*, 1995; Coll *et al*., 1995; Austin, 1998; Adams, 1999; Adams, 2004; Basurco and Toranzo, 2004; Adams and Thompson, 2006). The production and evaluation of a wide range of polyclonal and monoclonal antibodies have been reported (Coll *et al*., 1995; Romestand *et al*., 1995; Scapigliati *et al*., 1999; Adams, 2004; Jang *et al*., 2004; Basurco and Toranzo, 2004; Vesely *et al*., 2006) and a notable number of anti-species specific antibodies are currently commercially available for detection of fish pathogerns (Adams, 2004; Buller, 2004; Basurco and Toranzo, 2004) (Table 9.2) and monitoring the immune response of different fish species (Romestand *et al*., 1995; Scapigliati *et al*., 1999; Jang *et al*., 2004; Vesely *et al*., 2006) (Table 9.3). However, their application in commercial aquaculture systems is yet to be implemented (Basurco and Toranzo, 2004). Some reasons for the limited applications of the antibodies in past years are the rapid development of molecular techniques and their increased utilization in the detection and taxonomic characterization of microorganisms and the lack of standardised reagents and/or protocols for routine diagnose purposes (Basurco and Toranzo, 2004). The application of recombinant DNA technologies for antibody production and the combination of existing immunological and molecular techniques will provide powerfull tools

that can be applied for fish health management (Adams *et al.*, 2004; Cunnigham, 2002, 2004).

9.3 IMMUNOLOGICAL MEHODS COMMONLY USED IN AQUACULTURE

A variety of qualitative or quantitative immunological-based tests have been developed for detection of antigens or antibodies specific for bacterial diseases for clinical and veterinary medicine. These methods do not usually require extensive/destructive sample preparation or expensive reagents or instrumentation. The antibody-based assays most often used for the detection of bacterial fish pathogens are: agglutination test, immunofluorescence assays (direct and indirect), immunohistochemistry, enzyme immunoassays (ELISA) and dot blot (Adams, 1999; Adams, 2004; Adams and Thompson, 2006). New developed methods included test strips and multiplex assays based on Luminex technologies. All the methods have advantage and disadvantages and the choice of the appropriated tests for routine diagnosis will depend on a variety of factors including availability of reagents and standardised protocols, instrumentation, skilled operator to perform the assay and to interpret the results.

9.3.1 Agglutination Test

Agglutination tests are used to test an unknown organism against known antisera. Bacterial agglutination tests may be performed on a slide, in tubes or in microtitre tray wells. The slide agglutination tests are carried out by mixing antigen and serum on a plate and the reading of the presence of agglutination (visible precipitation of antibody-antigen complexes) by macroscopic examination, usually after 2 minutes incubation. Agglutination in tubes usually involves diluting the serum before the addition of the antigen. After 24 hours positive results are visualized by the presence of a precipitate in the bottom of the tube and a clearing of the supernatant (as compared to antigen without any serum). Micro-agglutination is carried out using relatively small amounts of antigen in a micro-titration plate, facilitating the examination of a large number of samples at various dilutions. In this case a positive reaction is manifested as a ragged blanket of antigen covering the bottom of the U-shaped micro-titre well, as opposed to a button of unreacted antigen in the negative wells. Tube and micro-agglutination tests are usually more sensitive than slide tests as they require a longer incubation period which

allows more antigen and antibody to interact. Attachment of antibodies to carrier particles such us latex vedas (so-called latex agglutination test) or intact formalkin-killed *Staphylococcus aureus* cells (co-agglutination test) increases the assay sensitivity. The agglutination test cannot be performed if the bacterial suspension is granular, autoagglutinates or is sticky. The agglutination techniques are widely used for identification and serotyping of bacterial pathogens such us non-autoagglutinating *Aeromonas salmonicida* (Rabb *et al*., 1964; Kimura and Yoshimizu, 1984; Toranzo *et al*., 1987, 1991), *A. hydrophila* (Santos *et al*., 1991), *Listonella anguillarum* (Sorensen and Larsen, 1986; Toranzo *et al*., 1987; Romalde *et al*., 1995; Santos *et al*., 1995; González *et al.*, 2004), *Photobacterium damselae* subspecies *piscicida* (Romalde *et al*., 1995), *Yersinia ruckeri* (Romalde *et al*., 1995), *Edwardsiella tarda* (Amandi *et al*., 1982), *Flavobacterium psychrophilum* (Santos *et al*., 1992; Lorenzen and Olesen, 1997; Izumi and Wakabayashi, 1999; Mata *et al.,* 2002), *Tenacibaculum maritimum* (Pazos, 1997; Santos *et al*., 1999; Avendaño *et al*., 2004) and *Renibacterium salmoninarum* (Kimura and Yoshimizu, 1981) by different laboratories (Basurco and Toranzo, 2004), policlonal and monoclonal antibodies as well as latex agglutination kits are commercially available for reliable identification of fish pathogens (Table 9.2, 9.3 and 9.4).

Table 9.2: Some polyclonal and monoclonal antibodies commercially available for diagnosis of bacterial fish diseases

Antibodies	**Specificity**	**Company**
Rabitt polyclonal	*T. maritimum* *P. damselae subsp. piscicida* *L. anguillarum serotype* O2 *V. ordali* *V. salmonicida* *Y. ruckeri serotype* O2 *R. salmoninarum* *S. iniae* *F. psychrophilum* *F. columnare*	Microtek International Inc.
Sheep polyclonal	*A. salmonicida* *L. anguillarum serotypes* O1 and O2 *V. salmonicida* *M. viscosa* *Piscirickettsia salmonis*	Microtek International Inc.
Goat polyclonal	*R. salmoninarum*	Microtek International Inc.

Mouse monoclonal	*P. damselae subsp. piscicida* *R. salmoninarum* *M. marinum* *S. iniae*	Microtek International Inc.

Microtek International Inc., 6761 Kirkpatrick Crescent, Saanichton, B.C., V8M 1Z8 Canada.

Table 9.3: Some monoclonal antibodies against fish immunoglobulins commercially availables

Antibody Specificity	Company
Atlantic salmon Ig	Microtek International Inc
Salmonid Ig	Microtek International Inc
Rainbow trout Ig	Microtek International Inc
European sea bass IgM	Microtek International Inc
Asian sea bass	Microtek International Inc
Sea bream IgM	Microtek International Inc
Tilapia IgM	Microtek International Inc
Grouper IgM	Microtek International Inc
Turbot IgM	Microtek International Inc
Cod IgM	Microtek International Inc
Halibut IgM	Microtek International Inc
Japanese Flounder IgM	Microtek International Inc
Dab IgM	Microtek International Inc

Microtek International Inc., 6761 Kirkpatrick Crescent, Saanichton, B.C., V8M 1Z8, Canada.

9.3.2 Immunofluorescence Assays

In these assays specific monoclonal or polyclonal antibodies conjugated with a fluorescent dye (usually fluorescein isothiocyanate) are used as reagent. In a direct fluorescent antibody test (FAT) the pathogen-specific antibody is labeled with the dye while in the indirect fluorescent antibody test (IFAT) a secondary species-specific antibody is labeled with the fluorescent dye instead of the pathogen-specific antibody. In immunoassay tests, a specimen is incubated with the fluorescent labeled antibodies and then visualized using a fluorescence microscope, fluorometer, fluorescence scanner or flow cytometer. FAT and IFAT are simple, fast and sensitive methods but has the disadvantage of requires specialized equipment and skilled operators (Adams and Tompson, 2006). Indirect and direct fluorescent antibody technique are usefull for

diagnosis of bacterial kidney disease (BKD) and piscirickettsiosis (Stevenson, 1999; OIE, 2006).

9.3.3 Immunohistochemistry

Immunohistochemistry (IHC) is a method of detecting the presence of the pathogen in cells or tissues. The tissue sample should be formalin-fixed, paraffin-embedded, deparaffinised, and treated to eliminate endogenous peroxidase and then incubated with a pathogens-specific antibody. The antibody-antigen complex is then bound by a secondary enzyme-conjugated antibody that in the presence of chromogen, forms a colored deposit at the sites of antibody-antigen binding that can be observed using a light microscope. Although is a method less sensitive than IFAT, IHC do not requires specialized equipment to read the results. IHC are usefull for diagnosis of piscirickettsiosis (Stevenson, 1999; OIE, 2006), vibriosis caused by *Vibrio salmonicida* (Evensen *et al*., 1991), pasteurellosis by *P. damselae* subsp. *piscicida* (Maniatis *et al*., 2000) and enteric redmouth disease (Jansson *et al.,* 1991).

9.3.4 Enzyme Liked Immunosorbent Assays (ELISA)

ELISA offers easy and non-hazardous detection of antigens or antibodies in tissues or serum of diseased animals. Although regularly used in human and veterinary medicine, detection of antibodies in the serum of fish is not usually employed to evaluate the health status of the animals (Adams and Thompson, 2006). The most common ELISA format used for the detection of fish pathogen is the capture (sandwich) ELISA. In this assay the samples are added to the ELISA plate coated with an antigen-specific antibody and, after washing to remove unbound material, a secondary antigen-specific antibody is added. In a direct format an enzyme is conjugate to the secondary antibody while in the indirect format the second pathogen-specific antibodiy is not conjugated and a third enzyme conjugated species-specific antibody is added to obtain the color reaction after the addition of substrate. The sensitivity of this method is relatively high and close to one step PCR and can be increased by using the avidin-biotin system. ELISA assay and ELISA-based assays are usefull tools for diagnosis of bacterial kidney disease (Hsu *et al*., 1991; Stevenson, 1999), *Photobacterium damsela* subsp. *piscicida* (Bakopoulos *et al*., 1997; Romalde *et al*., 1999), *A. hydrophila* (Sendra *et al*., 1997), *A. salmonicida* (Austin *et al.*, 1986; Hiney *et al.,* 1994), *V. vulnificus* (Biosca *et al*., 1997), *Y. ruckeri* (Cossarini-Dunier, 1985), *F. psychrophilum* (Rangdale and Way, 1995; Mata and Santos, 2001; Mata

et al., 2002) and are routinely sed by some diagnostic laboratories (Basurco and Toranzo, 2004). Monoclonal and policlonal antibodies to prefrom ELISA assays and ELISA-based kits are commercially available for the diagnosis of some bacterial fish diseases (Table 9.4).

Table 9.4: Commercial available test s for detection and serological identification of fish pathogens

Test	**Based Method**	**Diagnostic application**	**Company**
Bionor Mono Aqua			
Mono-Va (*L. anguillarum* serotypes O1, O2, O3, O4, O5 and O7	Agglutination test	At laboratories	Bionor
Mono-Vs (*V. salmonicida*)	Agglutination test	At laboratories	Bionor
Mono-As (*A. salmonicida*)	Agglutination test	At laboratories	Bionor
Mono Pp (*P. damselae* subsp. *piscicida*)	Agglutination test	At laboratories	Bionor
Mono Yr (*Y. ruckeri*)	Agglutination test	At laboratories	Bionor
Mono-Rs (*R. salmoninarum*)	Agglutination test	At laboratories	Bionor
Mono-Mv (*Moritella viscosa*)	Agglutination test	At laboratories	Bionor
Bionor Aquarapid			
Aquarapid-Va (*L. anguillarum* O1,O2, and *V. ordalii*)	ELISA test	At laboratories	Bionor
Aquarapid-Pp (*P. damselae* subsp. *piscicida*)	ELISA test	At laboratories	Bionor
Bionor Aquaeia			
Aquaeia-Va *(L. anguillarum* O1,O2, and *V. ordalii*)	Magnetic particles- ELISA test	At laboratories and fish farms	Bionor
Aquaeia-Pp (*P. damselae* subsp. *piscicida*)	Magnetic particles- ELISA test	At laboratories and fish farms	Bionor
Bead agglutination			
ADVA01 (*L. anguillarum* serotype O1)	Agglutination test	At laboratories	Microtek Int.
ADVA02 (*L. anguillarum* serotype O2)	Agglutination test	At laboratories	Microtek Int

ADVS01 (*V. salmonicida*)	Agglutination test	At laboratories	Microtek Int
ADVV01 (*M. viscosa*)	Agglutination test	At laboratories	Microtek Int.
ADYR01 (*Y. ruckeri* serotype O1)	Agglutination test	At laboratories	Microtek Int.
ADYR02 (*Y. ruckeri* serotype O2)	Agglutination test	At laboratories	Microtek Int.
Sanogurad Test strips			
D-VIB (*V. harvey* and *V. campbelli*)	Immunochromatography	At laboratorios and Fish Farms	Inve Aquaculture

Bionor Laboratories AS P.Box 2870 NO-3702 SKIEN – NORWAY, INVE TECHNOLOGIES nv Hoogveld 93 9200 DENDERMONDE BELGIUM; Microtek International Inc. 6761 Kirkpatrick Crescent, Saanichton, B.C., V8M 1Z8, Canada.

9.3.5 Dot Blot

Dot blot is a quick, convenient and relatively cheap method to detect the pathogens in tissue homogenate and for bacterial identification and serotyping. Nitrocellulose membrane is the common solid matrix for sample adsorption. The sensitivity of these method is similar to the ELISA and ELISA-based assays (Santos *et al*., 1995; González *et al*., 2004). The Dot blot assay is a potent tool for diagnosis of diseases caused by *L. anguillarum* and *V. ordalii* (Cipriano *et al*., 1985; Santos *et al*., 1995; González *et al.*, 2004), *A. salmonicida* (El Morabit, 1999; El Morabit *et al.,* 2003), *E. tarda* (Swain *et al*., 2001) and *T. maritimum* (Pazos, 1997; Avendaño *et al*., 2004).

9.3.6 Immunochromatographic Test Strip

This method requires a MAb conjugated to a laex or colloidal metal particle (detection antibody) laid on the sample pad, a second antibody (capture antibody), which may be MAb or polyclonal antibody, immobilized onto the surface of a porous membrane (nitrocellulose, nylon or teflon) and Goat anti mouse Ig (control line) on the nitrocellulose next to the capture antibody. When a sample is added to the conjugate pad, the detector reagent is solubilized and begins to move with the sample flow front up the membrane strip. The antigen in the sample is bound by the detection antibody and when the sample passes over the zone to which the capture antibody was immobilized the complex antigen-detector antibody is trapped and a color develops in proportion to the antigen present in the sample. This method is simple, quick, and easy to perform and no requires special equipment or skillful personnel to interpret the results. However, the sensitivity of this method

is still limited, generally close to dot blot method. The usefulness of this metdod for the detection of *V. harveyi* in pond water and infected shrimp has been reported (Sithigorngul *et al*., 2007). Currently, a strip test is commercially available for the detection of *V. harveyi* and *V. campbelli* in shellfish and fish tissues (Table 9.4).

9.3.7 Multiplex Assays Using Luminex Technology (Liquid Chip)

Luminex multiplex assay uses the 'sandwich' assay format, but in this system the capture antibody is coated onto the surface of polystyrene color coded bead that can be differentiated by an Luminex analyzer. Each type of bead can be labeled with different antibodies, this labeling enables multiple analyses to be simultaneously performed in the same well/tube. The beads are maintained in solution throughout the assay, thereby permitting liquid phase binding of target and separation spectrally by a dual laser detection system. The laser detects excitation of internal bead dyes and reporter dyes of any captured targets. The signal intensity is proportional to the amount of target analyte in the sample. The test is under development for the simultaneous detection and identification of a variety of bacterial and viral fish pathogens (Adams and Tompson, 2006). The Luminex system is a rapid, flexible platform capable of simultaneous, sensitive and specific detection of pathogens.

9.4 CONCLUSION

The development of non-culture based detection techniques for reliable detection/identification and epidemiological typing of pathogenic bacteria in diseased and carrier fish or in other reservoirs in aquatic environment is necessary for effective management of infection and understanding of disease etiology. Immunochemical methods offer simple, rapid, specific and sensitive tools for diagnosis based on the detection of antigens specific for bacterial diseases. Monitoring the fish antibodies to specific pathogens or the immune response of fish after vaccination has also potential as a mean of disease surveillance in aquaculture. In order to be adopted as routine method for the diagnosis of diseases in aquaculture, immunochemical methods should be standardized and validated in the field.

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10

Masculinization of Torafugu, *Takifugu rubripes* by Modulation of Aromatase Activity

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10.1 INTRODUCTION

Over the last few decades, the diminishing rate of natural fish stock and increasing rate of fish consumption by the increasing population have given rise to intensive aquaculture practices in both developing and developed worlds. Collection from the nature was the only source of fish seeds for aquaculture for many years. The unavailability of natural seeds and high demand in the increasing aquaculture practice has encouraged the scientists to develop fish seed production methods in captivity. Over the development of aquaculture as an industry, there has been an increasing demand for monosex population in aquaculture due to good production, taste and marketability of particular sex of particular species (Mair *et al.*, 1997; Shelton, 2002). Application of steroid hormones in developing fish has been the popular method of treatment for producing monosex population (Feist *et al.*, 1995; Kitano *et al.*, 2000; Papoulias *et al.*, 2000; Van den Hurk *et al.*, 1989). In many fish species, male has been found to grow faster than female and has good marketability, and androgenous steroids have been found to be popularly used for masculinizing them (Bhandari *et al.*, 2006; Wassermann and Afonso, 2003). In some cases, these androgenous steroids have been found to result in the development of phenotypic female fish instead of male, a phenomenon, which is described as ‘paradoxical feminization’ (Hackman

and Reinboth, 1974; Hackman, 1971; Tiwary *et al.*, 1998). Poor performance of androgen has also been observed in the process of masculinization of some fishes leading to mixed sex population (Gale *et al.*, 1999; Komen *et al.*, 1989). Moreover, the application of sex steroids on fish has been restricted in many countries due to health concern. An alternative way of masculinizing genetic female fish has been the use of non-steroidal compounds with potent ability to block synthesis of estrogens, steroid hormones responsible for femaleness. These compounds, called aromatase inhibitors (AIs), can modulate aromatase which is responsible for synthesis of estrogen from androgen in most of the vertebrates (Callard, 1981; Kellis and Vickery, 1987; Nelson *et al.*, 1993; Osawa *et al.,* 1987). In many teleosts, these AIs have been found to potentially inhibit circulating estradiol-17β (E2) production by blocking the activity of aromatase and resulting in masculinization of genetic female fish (Ankley *et al.*, 2002; Bhandari *et al.*, 2004; Fenske and Segner, 2004; Mandiki *et al.*, 2005; Tzchori *et al.,* 2004).

In this chapter, we have discussed the effects of non-steroidal AI (here fadrozole) in regulating sexuality parameters of fish using Japanese pufferfish (*Takifugu rubripes*), popularly called torafugu, as a test animal. We have described the consequences of modulation of aromatase on torafugu during the crucial period of gonadal sex differentiation. Moreover, the importance of torafugu as a model species for studying reproductive biology and sexuality of teleosts has been discussed. Most of the scientific findings on torafugu, provided in this chapter, have been obtained from our previous investigations done on this species.

10.2 TORAFUGU: A MODEL SPECIES FOR STUDYING SEXUALITY PARAMETERS

The torafugu, *T. rubripes*, is a gonochoristic tetraodontiform fish, which lives in the marine waters of Japan, Korea and China. It is the second vertebrate (the first being human) whose genome has been completely sequenced and whose genome is among the smallest vertebrate genomes, only 390 Mb. Torafugu genome has proved to be a valuable reference genome for identifying genes and other functional elements such as regulatory elements in the human and other vertebrate genomes, and for understanding the structure and evolution of vertebrate genomes (Aparicio *et al.*, 2002; Brenner *et al.*, 1993). As a part of understanding the mechanism of gonadal sex determination, differentiation and development of torafugu, we have previously carried out the histological

investigation of the process of gonadal sex differentiation and the course of ovarian and testicular development in this species was identified (Matsuura *et al.*, 1994; Yamaguchi *et al.*, 2006). Conventional histological analysis in these studies showed that a primordial gonad is present 2 weeks after hatching, and most importantly, sexual differentiation had occurred by 6-weeks after hatching (i.e., 42-days after hatching, dah), determined by the formation of cavities in the ovaries. Gonadal sex differentiation in torafugu continues upto around 3-months after hatching (mah) indicated by proliferation of spermatogonia and oogonia at this stage. In a recent study with torafugu DMRT1 (*Doublesex/mab3* related transcription factor 1), a transcription factor that regulates early differentiation of Sertoli cells in the testis of vertebrates, we have found that it is involved in gonadal development and that its expression has been correlated with the proliferation of spermatogonia (Yamaguchi *et al.*, 2006). Moreover, we have developed the maturation- and ovulation-induction method in cultured torafugu by hormonal treatment (Chuda *et al.*, 1996). Our investigations on the effects of environmental parameters (e.g., temperature) on the sexuality of this fish revealed that high temperature (29°C, environmental water temperature being 17°C) has no effect on sexuality of this fish. Recently, by performing genomewide linkage analyses, Kikuchi *et al.* (2007) revealed that the sex of torafugu is determined by a single region on linkage group 19 in an XX–XY system. Further investigations on the molecular and endocrine factors related to sexuality of this species are underway in our research. The advantage of knowing the genome sequence and availability of information on the gonadal sex differentiation and development conducted in our previous works have made torafugu an important model species for studying factors related to sexuality of teleosts.

10.3 AROMATASE AND ITS ISOFORMS IN TORAFUGU

Aromatase cytochrome P450 (P450arom, the product of the CYP19 gene) is a microsomal member of the cytochrome P450 superfamily which catalyzes the biosynthesis of estrogen in animals. The P450 gene superfamily contains over 480 members in 74 families, of which cytochrome P450arom is the only member of family 19 (Nelson *et al.*, 1996). This heme protein is responsible for binding of the C19 androgenic steroid substrate and catalyzing the series of reactions leading

to formation of the phenolic A-ring characteristic of estrogens (Kellis and Vickery, 1987; Mendelson *et al.*, 1985; Nakajin *et al.*, 1986; Nelson *et al.*, 1993; Osawa *et al.*, 1987; Thompson and Siiteri, 1974;). The reducing equivalents for this catalytic reaction are supplied from NADPH *via* a microsomal flavoprotein, NADPH-cytochrome P450 reductase. In teleost fishes, a number of tissues have been found to exert the capacity to express aromatase and hence synthesize estrogens. In most of the teleosts, aromatase isoforms have been found to be expressed mainly in brains, ovary and testis. Other than teleosts, the biosynthesis of estrogens appears to occur throughout the entire vertebrate phylum including mammals, birds, reptiles, amphibians, elasmobranch fish, and agnaths (Callard *et al.*, 1978, 1980; Callard, 1981).

Analysis of CYP19 gene function has been carried out in several fish species including torafugu. Like in most of the teleosts, in torafugu, there are two forms of aromatase predominantly expressed in bran and ovary and are termed as brain type (pfCYP19B) and ovarian type aromatase (pfCYP19A) (Chang *et al.*, 2005; Goto-Kazeto *et al.*, 2004; Kitano *et al.*, 1999; Luckenbach *et al.*, 2005; Rashid *et al.*, 2007a; Strobl-Mazzulla *et al.*, 2005).

10.3.1Role of Brain Type Aromatase in Gonadal Sex Differentiation and Development of Torafugu

Like in most of the teleosts, torafugu brain type aromatase (pfCYP19B) is predominantly expressed in brain. We have found that the torafugu brain type aromatase has a single CYP19B gene encoding two transcripts of different sizes (the shorter transcript, pfCYP19B-I and the longer transcript, pfCYP19B-II). The occurrence of multiple transcripts of CYP19B in fish and other animals are very rare and rainbow trout and channel catfish furnish two such rare examples (Dalla Valle *et al.*, 2005; Kazeto and Trant 2005). Common to the expression pattern in many of the teleosts (Chang *et al.*, 2005; Fenske and Segner, 2004; Fukada *et al.*, 1996; Kishida and Callard, 2001; Trant *et al.*, 1997), pfCYP19B-I is highly expressed in torafugu brain of both sexes throughout the life. In addition, an exceptionally high level of pfCYP19B-I is found in testis upto immature stage and pfCYP19B-II at mature spermiating stage. The high expression of torafugu brain type aromatase in testis is a quite exceptional case in any fish and there is no such report of high CYP19B expression in the testis of any teleosts studied so far. This high pfCYP19B expression in testis suggests an important role of brain type aromatase in testicular maturation in torafugu. Moreover, our recent *in situ*

hybridization investigations of the two pfCYP19B transcripts in torafugu testis at immature and maturity stages gives clarity of this speculation. The pfCYP19B-I localization found in somatic cells inside the immature torafugu testis and pfCYP19B-II localization in spermatids suggest important roles of pfCYP19B in testicular cell proliferation and germ cell maturation (Rashid *et al.*, 2007a).

10.3.2 Role of Ovarian Type Aromatase in Gonadal Sex Differentiation and Development of Torafugu

We have shown that ovarian type aromatase (pfCYP19A) mRNA is found to be expressed in torafugu during larvalhood and early development. The mRNA expression pattern revealed that at early stages, pfCYP19A is not specific to particular tissue types and found to be expressed overlapping in both gonad and brain; whereas at immature and mature stages, the expression is almost ovary-specific. The highest level of pfCYP19A expression was evident in previtellogenic ovary. Ovarian pfCYP19A expression is low in fishes, which have almost reached the end of vitellogenesis when the need for estrogen synthesis is decreased. pfCYP19A expression in testis is also low and not statistically different at different stages of testicular development and in reproductively mature spermiating testis the expression is almost non-detectable. The trend of pfCYP19A expression in brain shows a very low activity in this tissue type throughout the life (Rashid *et al.*, 2007a).

10.4 AROMATASE INHIBITOR TREATMENT AND MODULATION OF AROMATASE ACTIVITY

In the previous sections we have discussed the role of two aromatase isoforms in torafugu and found that pfCYP19A and pfCYP19B play important role in gonadal development. In the current section, the role of a non-steroidal aromatase inhibitor, fadrozole, in regulating abundance of these two pfCYP19 isoforms and resultant alteration in steroid synthesis and gonadal differentiation are described.

10.4.1 Aromatase Inhibitor Results in Suppression of Aromatase (CYP19) mRNA Species

The aromatase inhibitor, fadrozole, has shown to reduce the activity of aromatase and therefore E2 formation in gonad and brain of mammals (Steele *et al.*, 1987; Harada *et al.*, 1999), birds (Foidart *et al.*, 1995;

Wade *et al.*, 1994) amphibians (Chardard and Dournon, 1999; Miyata and Kubo, 2000) and fishes (Afonso *et al.*, 1997, 1999, 2001; Ankley *et al.*, 2002; Lee *et al.*, 2001). In zebrafish and red-spotted grouper, for example, aromatase inhibitor has been found to suppress CYP19 gene expression resulting in masculinization of female fish (Fenske and Segner, 2004; Li *et al.*, 2006). In our recent investigation on CYP19 regulation and AI-induced testicular differentiation of torafugu, fadrozole has been found to suppress both forms of pfCYP19 species in brain and gonads, tissues with potent local steroid synthesis ability (Rashid *et al.*, 2007a). AI-treatment inhibited pfCYP19A mRNA in gonads during the crucial period of ovarian cavity formation and pfCYP19B in gonad and brain by the end of gonadal sex differentiation. Both pfCYP19A and pfCYP19B were upregulated (towards normalization) after withdrawing fadrozole application.

10.4.2 Aromatase Inhibitor Results in Upregulation of Androgen and Downregulation of Estrogen Production

In higher vertebrates like mammals (e.g., monkey, hamster, rabbit and rat), fadrozole treatment results in reduced E2 production (Moudgal *et al.*, 1996; Shetty *et al.*, 1995). In fish also, fadrozole is reported to inhibit E2 production (Bhandari *et al.*, 2004; Mandiki *et al.*, 2005) resulting in masculinization of genetic female fish (Afonso *et al.*, 2001; Fenske and Segner, 2004; Kitano *et al.*, 2000; Kwon *et al.*, 2000; Tzchori *et al.*, 2004). In our recent investigation on fadrozole effects on circulating androgen–estrogen balance of torafugu (Rashid *et al.*, 2007b), low estrogen (E2) and high androgen (11-ketotestosterone, 11-KT; and testosterone, T) levels were evident in AI-treated fish throughout the experimental period (Figure 10.1). After withdrawing fadrozole exposure, there were no significant differences of E2 levels in immature torafugu (9-mah) between control and treatment although the highest level was detected in control female. By the end of the fadrozole exposure period, 11-KT levels in treated fishes were almost twice as much as in control males. The 11-KT level decreased in immature AI-treated torafugu when it had no significant difference with control male. The highest level of steroid detected in treated fish during AI exposure period was T, as observed in AI-treated fish on 100-dah. This T value was almost double the value in control male at this stage. After withdrawing fadrozole application, there was a significant drop of T level in both AI-treatments towards normalization. The steroid profile in AI-treated

torafugu indicates that fadrozole can potentially block aromatase activity resulting in low circulating E2 and high T.

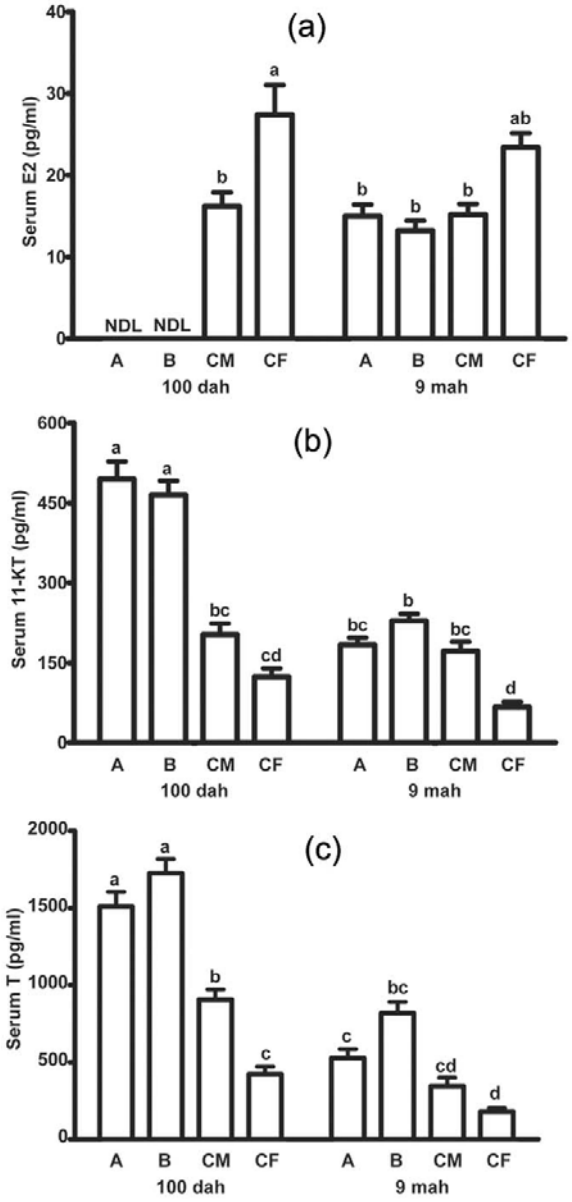

Figure 10.1: Effects of aromatase inhibitor on the circulating steroid production of torafugu [measured on 100-dah and 9-mah]. (a) Serum estradiol-17β (E2), (b) serum 11-ketotestosterone (11-KT), and (c) serum testosterone (T). A, fadrozole treatment A (500μg/g diet); B, fadrozole treatment B (1000 μg/g diet); CM, control male; CF, control female. Values above the bars indicate significant differences

($p<0.05$) compared within the data analyzed by one-way ANOVA followed by Tukey's multiple comparison test. ELISA was performed according to the protocol described by Matsuyama et al. (1998).

10.4.3 Aromatase Inhibitor Results in Inhibition of Ovarian Cavity Formation and Masculinization of Genetic Female Fish

Aromatase inhibitor, fadrozole, has been found to result in inhibition of ovarian cavity formation, formation of intermediate gonad, and finally masculinization of genetic female in both gonochoristic and hermaphroditic fishes (Afonso *et al.*, 2001; Fenske and Segner, 2004; Komatsu *et al.*, 2006; Kwon *et al.*, 2000; Suzuki *et al.*, 2004; Tzchori *et al.*, 2004). Not only fadrozole but also other AIs and exogenous androgens were found to influence masculinization in fishes (Feist *et al.*, 1995; Kitano *et al.*, 2000; Piferrer *et al.*, 1994). As an effect of fadrozole treatment, inhibition of ovarian cavity formation and a gradual trend towards masculinization was observed in all AI-treated torafugu. Our recent histological investigation on AI-treated torafugu gonads (Rashid *et al.*, 2007b) revealed that, on 42-dah (the crucial period of morphological gonadal sex differentiation of torafugu according to Yamaguchi *et al.*, 2006), non-treated fishes were composed of female and male individuals as identified by ovarian cavity (ovary) formation and no ovarian cavity (testis) (Figure 10.2a and 10.2b). In both the AI-treatments (where treatment 'A' is 500 μg fadrozole/g diet and 'B' is 1000 μg/g) some of the gonads contained deformed ovarian cavity (Figure 10.2c) compared to the well-developed ovarian cavity in control ovary (Figure 10.2b), and rest of the gonads resembled control testis (arrowheads in Figure 10.2a). On 70-dah, the control ovary had well-developed ovarian cavity and proliferated oogonia (Figure 10.2e). Control testis was composed mostly of spermatogonia (Figure 10.2d). On the other hand, AI-treated gonads were composed of testis similar to control and intermediate gonads having deformed ovarian cavity and empty lumina of tubules (Figure 10.2f). The intermediate gonads were filled with gonia, which were not histologically distinguishable from spermatogonia and oogonia.

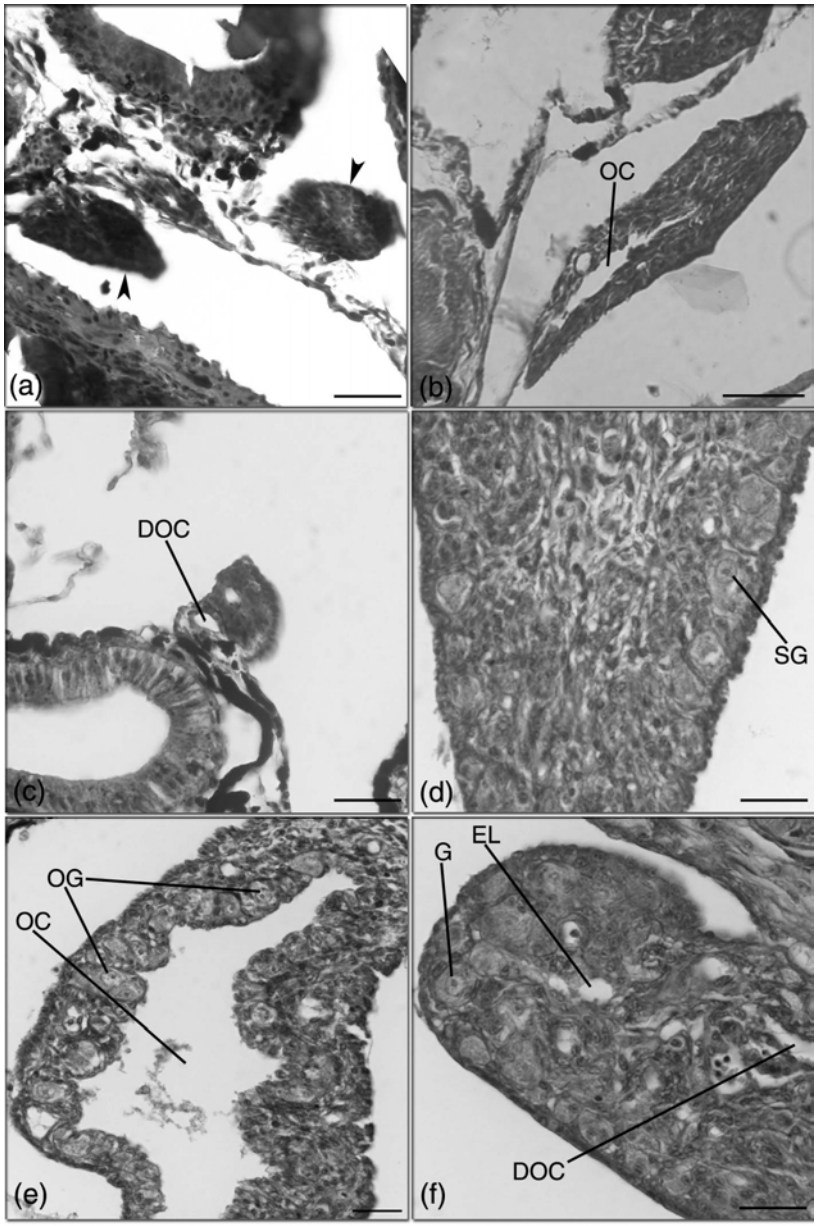

Figure 10.2: The histo-photographs of AI-induced masculinization process as evident in torafugu gonads from 42-dah to 70-dah. (a) Control testis on 42-dah (arrowheads); (b) control ovary on 42-dah; (c) intermediate gonad from fadrozole treated fish on 42-dah; (d) control testis on 70-dah; (e) control ovary on 70-dah; (f) intermediate gonad from fadrozole treated fish on 70-dah. OC, ovarian cavity; DOC, deformed ovarian cavity; OG, oogonium; SG, spermatogonium; G, gonium; EL, empty lumini of tubule. Scale bars, 20-µm.

Gonadal histology by the end of the sexual differentiation period (on 100-dah; termination of fadrozole treatment) revealed that control ovary contained cavity and composed of oogonia and perinucleolar oocytes (Figure 10.3b). Control testis, on the other hand, had proliferating spermatogonia (Figure 10.3a). AI-treated gonads were composed of testis similar to control and intermediate gonads containing deformed ovarian cavity and remained oocytes (Figure 10.3c). Histology of gonads well after withdrawal of AI-treatment (investigated on 6-mah) revealed that all testes (both treatment and control) were found to be undergoing spermatogenesis. Spermatogonia, spermatocytes and spermatids were observed in histological sections (Figure 10.3d). All the AI-treated gonads resembled control testis. On the other hand, control ovary was consisted mostly of perinucleolar oocytes and previtellogenic oocytes (Figure 10.3e). Investigation of gonads at immature stage (9-mah) revealed that in control and AI-treated testes, the final stage of spermatogenesis, spermatozoa, were observed in histological sections together with spermatogonia and spermatocytes (Figure 10.3f). On the other hand, in the control ovary, cellular stages were almost similar to that observed on 6-mah ovary. The gonad histology of AI-treated gonads clearly indicates that fadrozole treatment during gonadal differentiation can inhibit ovarian cavity formation and ultimately results in masculinization of all AI-treated torafugu.

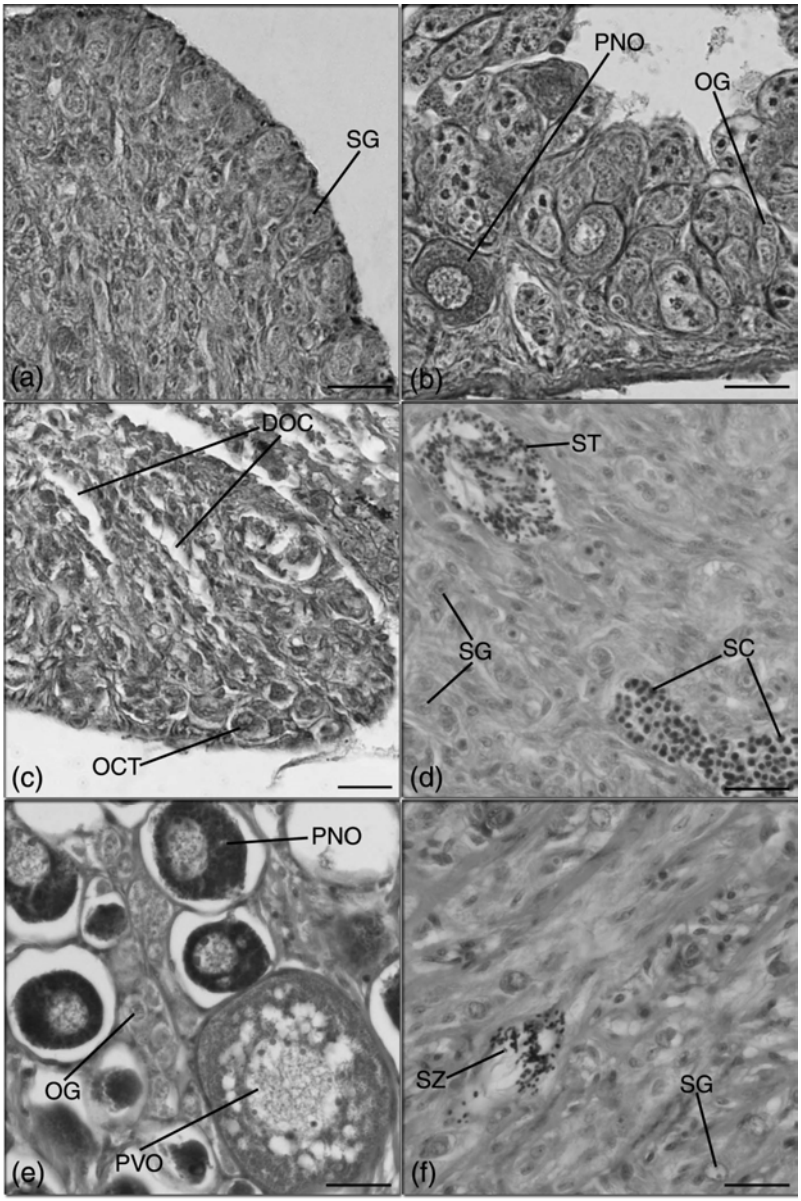

Figure 10.3: The histo-photographs of AI-induced masculinization process as evident in torafugu gonads from 100-dah to 9-mah. (a) Control testis on 100-dah; (b) control ovary on 100-dah; (c) intermediate gonad from fadrozole treated fish on 100-dah; (d) testis from fadrozole treated fish on 6-mah; (e) control ovary on 6-mah; (f) testis from fadrozole treated fish on 9-mah. OC, ovarian cavity; DOC, deformed ovarian cavity; OG, oogonium; OCT, oocyte; PNO, perinucleolar oocyte; PVO, previtellogenic oocyte; SG, spermatogonium; SC, spermatocyte; ST, spermatid; SZ, spermatozoon. Scale bars, 20-µm.

10.5 THE MASCULINIZATION MECHANISM

The role of androgen and estrogen in fish in inducing ovarian and testicular differentiation, respectively, has been proposed for the first time by Yamamoto (1969) from the results of his pioneering works on medaka. Since then, many studies have supported Yamamoto's prediction on the key function of sex steroids in sexual differentiation of fish (Baroiller *et al.*, 1999; Guiguen *et al.*, 1999; Nakamura *et al.*, 1998; Pandian and Sheela, 1995). In the process of gonadal sex differentiation and development of fish, the balance between estrogens and androgens appears to be crucial, and this balance depends upon the availability and activity of the steroidogenic enzymes, in particular on the cytochrome P450 aromatase complex. The importance of aromatase in gonadal sexual differentiation in fish is shown by the fact that modulation of the aromatase activity by using aromatase inhibitor during the crucial period of gonadal sex differentiation can cause masculinization of genetic female fish (Kitano *et al.*, 2000; Kroon and Liley, 2000; Piferrer *et al.*, 1993; Rashid *et al.*, 2007b).

In teleosts, the mechanism of AI-induced inhibition of ovarian cavity formation and resultant masculinization through disruption of aromatase function has been found to be controlled in different ways in different species. In zebrafish, for example, irreversible masculinization was found to be achieved by the manipulation of aromatase system by fadrozole during the critical period of sexual differentiation (Fenske and Segner, 2004). AI-induced sex inversion in red-spotted grouper was attributed to the inhibition of P450arom gene expression and aromatase activity and the resultant decrease in the biosynthesis of endogenous E2 (Li *et al.*, 2006). In the case of golden rabbitfish, estrogen was predicted to be involved in ovarian differentiation and AI-induced suppression of estrogen was found to be an essential prerequisite for masculinization (Komatsu *et al.*, 2006). In torafugu, similar to the studies mentioned above, AI-treatment during the period of gonadal sex differentiation was found to induce testicular differentiation towards irreversible masculinization.

In Figure 10.4, the mechanism of AI–CYP19–sex steroid interaction in the process of masculinization of torafugu has been illustrated. In the process of irreversible masculinization, importantly, the inhibition of ovarian cavity formation during the crucial period of gonadal sex differentiation was correlated with a massive suppression of pfCYP19A mRNA in AI-treated trunk at this stage suggesting that ovarian type aromatase plays an important role in torafugu ovarian cavity formation

and its suppression resulted in the inhibition of this process. Like in the case of torafugu, ovarian type aromatase has been found to play important role in the process of ovarian cavity formation of many teleosts (Devlin and Nagahama, 2002) including rainbow trout, Nile tilapia, European sea bass, medaka (Guiguen *et al.*, 1999; Kwon *et al.*, 2001; Piferrer *et al.*, 2005; Suzuki *et al.*, 2004). Different from the role of ovarian type aromatase, brain type aromatase was found to play important role in regulation of T–E2 balance in torafugu. This was evident by a massive suppression of pfCYP19B mRNA in AI-treated torafugu at the end of gonadal sex differentiation period and resultant high serum androgen (T and 11-KT) plus non-detectable limit of E2 at this stage. The suppression of pfCYP19B by AI-treatment at the end of the gonadal sex differentiation process and resultant imbalance in circulating sex steroids was probably responsible for AI-induced testicular differentiation of developing torafugu towards masculinization.

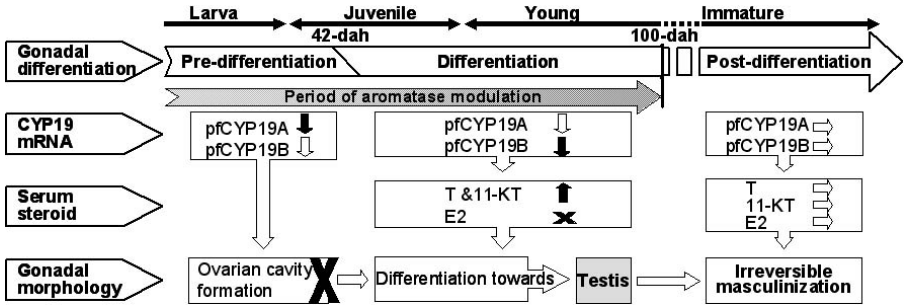

Figure 10.4: Diagrammatic representation of AI–CYP19–sex steroid interaction in the process of masculinization of torafugu. Top arrow shows different stages of gonadal sex differentiation: considering 42 dah as the point of morphological gonadal sex differentiation, 'pre-differentiation' refers to the stage where gonad is morphologically undistinguishable to testis or ovary; 'differentiation' refers to the period of gonadal sex differentiation and development from 42 dah; and 'post-differentiation' refers to the resting stage after gonadal sex differentiation is completed. From the highest to lowest degree of expression, small arrows inside the text boxes indicate: (⬆) upregulated level (⇨) normalized level; (⬇) lowered level; (⇩) suppressed level; (✖) inhibited level. dah, days after hatching; mah, months after hatching. Figure modified from Rashid et al. (2007a).

10.6 CONCLUSION

In this chapter, a brief overview of our study on the effect of an AI, fadrozole, on torafugu has been presented. Moreover, the effects of modulation of aromatase activity in other teleosts have also been discussed in relation to the case in torafugu. We have found that like in

many of the teleosts, treatment of torafugu with AI from 'first feeding' stage through the period of gonadal sex differentiation can block ovarian cavity formation of female fish followed by testicular differentiation. In torafugu, AI-induced suppression of pfCYP19A before gonadal sex differentiation resulted in the inhibition of ovarian cavity formation. Suppression of pfCYP19B by the end of gonadal sex differentiation and the resulting imbalance in circulating sex steroids contributed to the masculinization of all AI-treated torafugu. The compilation of molecular, endocrine and morphological evidences of masculinization mechanism of torafugu in this chapter can give important information for understanding the role of aromatase enzyme system in gonadal sex differentiation of teleosts. However, since the genetic marker for sex determination of torafugu is not identified yet, we could not confirm the genetic sex of the AI-treated masculinized fishes.

While torafugu is considered as an important model species for identifying genes and other functional elements in the human and other vertebrate genomes and for studying different molecular, transcriptional and endocrine factors of teleost reproduction, the amount of related scientific information are limited in the available literature. Moreover, rearing system for obtaining different developmental stages from embryo to adult is limited, and sometimes critical, for some model fish species with genome sequence information (e.g., *Tetraodon*). On the other hand, our previous researches (Chuda *et al.*, 1996; Matsuura *et al.*, 1994; Rashid *et al.*, 2007a; Yamaguchi *et al.*, 2006) have standardized the gonadal maturation, seed production, and developmental characteristics of torafugu (also discussed in previous sections of this chapter) which will provide guidelines for studying developmental biology and sexuality parameters of this model fish species in future.

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11

A Review of Grass Shrimp, *Palaemonetes pugio*, as a Biomonitor Species of Oxytetracycline Impacts

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11.1 INTRODUCTION

The current United States (US) system for assessing the risks of therapeutics is outdated and inadequate. The present system focuses primarily on the rare adverse effects of newly marketed drugs, because of regulatory requirements, rather than the much more common adverse effects due to the improper use of drugs. Risk assessment should continue throughout the life cycle of medical products, not just during initial development and use. Risk assessment must be conducted proactively, not just in response to crises (CERTs, 2003). Pharmaceuticals, hormones and other organic wastewater contaminants have been frequently reported in U.S. surface waters (Kolpin *et al*., 2002) as well as in Europe.

The Pharmaceutical Assessment and Transport Evaluation (PhATE) model is a tool developed by pharmaceutical companies to assess the potential risk of their products to human health and the environment. Based on the premise that patient use is the primary pathway to the environment, the model predicts exposure concentrations of pharmaceuticals in the environment and ultimately in drinking water. In view of the vast number of compounds that need to be assessed, simplicity and cost-efficiency, the model still uses a science-based approach (Anderson *et al*., 2004). This has been achieved by focusing on aquatic environments, where most substances may finally reside. Risk

assessments for these compounds should also employ the same representative target organisms (fish, crustaceans and algae) recommended for use in the assessment of other toxic chemicals. The importance of increasing our knowledge of the basic physiology of these compounds on aquatic organisms is of paramount importance as well as knowledge about the pharmacological effects in mammals as applied to aquatic organisms. Thus, it will become possible to predict effects in other species and to develop environmental endpoints to assess and study (The Swedish Association of the Pharmaceutical Industry, 2004).

In the U.S., the Environmental Protection Agency (EPA) is working with the United States Geological Survey (USGS) and the National Oceanic and Atmospheric Administration (NOAA) to develop a database of scientific literature on Pharmaceuticals and Personal Care Products (PPCPs). So far the database includes more than 400 peer-reviewed articles dating from the 1970's to present. Some view EPA's announcement that certain PPCPs may be placed on the 2008 Contaminant Candidate List (CCL) as evidence that the agency may be developing future regulations for these compounds. However, establishing reasonable regulatory limits is a challenge given that predicting the impact of each of these compounds on human health and the environment by studying the fate, transport, mode of action, and toxicity of each individual chemical is nearly impossible. Tens of thousands of PPCPs are currently being used by the public, with more coming to market each year. The task becomes even more daunting when dealing with complex mixtures of these chemicals that may be in various states of degradation or transformation, factors that operate differently depending on the specific environment, target species and each drug fate and effects in the environment (NACWA, 2005).

11.1.1 Antibiotics in the Environment

Aquaculture effluents are regulated under the National Pollutant Discharge Elimination System (NPDES) as part of the Federal Water Pollution Control Act of 1972 and its subsequent amendments However, the remarkable growth of the aquaculture industry since the 1980's has caused more and more states to consider developing regulatory statutes (SRAC, 1999). Furthermore, the recent discovery of pharmaceuticals across the USA has raised the visibility and need for monitoring of antibiotics in the environment (Thurman *et al.*, 2002) as well as the potential impact over sensitive species.

In a two-year study conducted in seven states in the US, Dietze *et al*. (2005) reported antibiotics in 15% of water samples collected during the first year and in 31% of the samples during the second year. Furthermore, they determined that ormetoprim, sulphadimethoxine and oxytetracycline concentrations were higher in samples from intensive (e.g. high stocking densities of organisms) aquaculture hatcheries (10, 12 and 36 μg/L), respectively, than in samples from the extensive (e.g. low stocking of organisms) aquaculture hatcheries (<0.05, 1.2 and 0.31 μg/L). These authors concluded that sulphadimethoxine persisted for a longer period of time (up to 48 days) than ormetoprim (up to 28 days) and oxytetracycline (less than 20 days). However, previous studies have demonstrated that antibiotics may be bound to the sediment (Bebak-Williams *et al*., 2002), posing chronic hazards to the environment. Consequently, more research is needed to better understand the processes and pathways of antibiotics and their degradation products in the sediment-interstitial water system.

The past two decades have also seen a dramatic rise in the number of concentrated animal feeding operations (CAFOs) and the subsequent increase in the use of veterinary antibiotics. While the number of domestic animals (chickens, cows and pigs) has remained relatively consistent, they are confined in concentrated areas intensifying the impact on water quality. The Union of Concerned Scientists (UCS, 2001) estimated that livestock producers in the US use 24.6 million pounds of antimicrobials, including antibiotics, annually for non-therapeutic purposes, such as to promote growth. In addition, antibiotics are also added to feed and water to reduce the losses of livestock to infectious diseases. Animals in confined production areas are more susceptible to disease when they are crowded together, prompting the need for antimicrobials.

11.1.2 Use of Oxytetracycline in Aquaculture

Oxytetracycline (OTC), derived from *Streptomyces rimosus,* is a broad-spectrum antibiotic from the tetracycline group. Its chemical formula is $C_{22}H_{24}N_2O_9$ (Figure 11.1). Oxytetracycline has four aromatic rings in its structure and crystalline bases, which are light yellow, odorless and bitter. OTC inhibits bacterial protein synthesis and the cellular site of action is the bacterial ribosome. Inside the bacteria cell, OTC binds principally to the 30S sub-unit of ribosomes; OTC prevents access of aminoacyl t-RNA to the acceptor site on the mRNA-ribosome complex, which blocks the addition of aminoacids to the growing peptide chain,

thus disrupting protein synthesis. Only a small portion of the drug is irreversibly bound and the inhibitory effects of OTC are reversible when the drug is removed (Sande and Mandell, 1985; Pelczar *et al*., 1997).

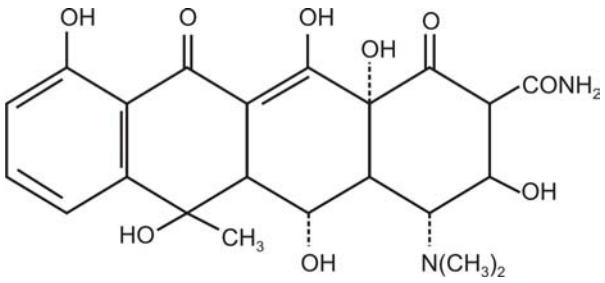

Figure 11.1 Molecular Structure of Oxytetracycline.

OTC was investigated for potential use in aquaculture shortly after its discovery and use in human medicine. In 1951, Samuelsen (1993) found that oxytetracycline could effectively be used against ulcer disease caused by *Haemophilus piscium* in brook trout (*Salvelinus fontinalis*). This led to OTC rapidly becoming the drug of choice in the treatment of bacterial diseases in fish. The recommended dosage of OTC for fish farming is between 80-100 mg/Kg of body weight per day for 8-10 days given as medicated food pellets (Pouliquen *et al*., 1992; Samuelsen, 1993).

A wide variety of chemotherapeutic agents, typically as medicated feeds, have been used in the aquaculture of finfish and lobsters, but no drugs have been approved for use in shrimp in the US (Schnick *et al*., 1986). Guest (1991) reported that 20% of the FDA animal drug requests were for aquaculture use. OTC is commonly used to combat bacterial infections in fish farming, and it has potential for use in farm-raised shrimp for the treatment of Vibriosis and necrotizing hepatopancreatitis infections. In shrimp producing countries like Taiwan, Thailand, Mexico, China, Brazil and Ecuador, OTC dose levels range from 1500 to 5000 mg/Kg of feed for 7-14 days (Montoya and Reyes, 2001; Uno *et al*., 2006, Nogueira-Lima *et al*., 2006). Although OTC has not been appoved in the U.S. for use in shrimp feed, OTC is used to treat farmed shrimp under an Investigational New Animal Drug (INAD) exemption, allowing research on unapproved drugs (Reed *et al*., 2006).

11.1.3 Potential Effects of Oxytetracycline

OTC exposure levels have been estimated based on concentrations of antibiotic in food pellets and assume a 50-100% loss in the water

(Arango *et al*., 1996; Montoya and Reyes, 2001). Exposure levels may vary based upon the physical-chemical conditions of the parent compound (Smith, 1996). In the US, in a study conducted in 30 states in 1999-2000 (Kolpin *et al*., 2002) found levels of OTC in streams at concentrations ranging from 0.10-0.34 μg/L. Moreover, Thurman *et al*. (2002), reported antibiotics in effluents from fish hatcheries, where OTC values in the water column ranged from 0.17-10 μg/L. Systems using recirculating water supplies may observe substantially higher OTC concentrations than in the adjoining surface waters (Bebak-Williams *et al*., 2002). Rose and Pedersen (2005) found that 10 to 15% of administered OTC would be released as a pulse to aquaculture surface water during initial treatment and for the first five days thereafter.
Persistent antibiotic residues could also promote antibiotic resistance among native bacteria in the environment. Tetracycline resistant bacteria are quite common and may accumulate near aquaculture hatcheries and facilities (Vaughan *et al*., 1996; Tendencia and de la Peña, 2001; Miranda and Zemelman, 2002; Tendencia and de la Peña, 2002). Hansen *et al*. (1992) found that the total number of bacteria in sediments, with added antibiotics like oxytetracycline, oxolinic acid and furazolidone, decreased by 50-70% a few days after the antibacterial agents were initially added to the sediments. Sulfate bacteria reduction rates were inhibited by less than 10%, but returned to normal levels after 70 days. Torkildsen *et al*. (2000) reported Minimum Inhibitory Concentration (MIC) values ranging from 0.5 to greater than 16 μg/mL for florfenicol in marine bacteria isolated from scallop hatchery larvae.

Various studies in both laboratory and field conditions have examined the fate and effect of oxytetracycline in the environment. Most of these studies have focused in the sediments given that as much as 95% of the antibiotic in feed can be released to the environment as the parent compound (Pouliquen *et al*., 1996; Rose and Pedersen, 2005). Most of these investigators seem to have accepted that deposition in the sediments has been the most important possible fate of OTC. Other researchers have measured OTC concentrations in sediment ranging from 0.4-419 μg/g sediment (Møster, 1986, Samuelsen *et al*., 1992; Capone *et al*., 1996).

11.2. GRASS SHRIMP, *PALAEMONETES PUGIO*

The grass shrimp, *Palaemonetes pugio*, is the predominant pelagic crustacean macrofauna found in estuarine tidal creek ecosystems of the

southeastern Atlantic and Gulf coast of the U.S., accounting for > 56% of total macro-faunal abundance on an annual basis. *Palaemonetes pugio* is a major component in the energy cycle of estuaries, primarily by eating and breaking down large amounts of dead *Spartina* into detritus (Welsh, 1975). Grass shrimp are very important in estuarine food webs as a primary food source by many fish species and they have been used to assess the toxicity of numerous contaminants (Key *et al*., 2006).

11.2.1 Use of *P. pugio* in Toxicity Tests

The grass shrimp has been used as a bioassay organism since 1975 (American Public Health Association). *P. pugio* is vulnerable to chemicals including organophosphorus insecticides, organochlorine insecticides and synthetic pyrethroid insecticides (Key, 1995; Leight *et al*., 2005). Several studies have been conducted with different grass shrimp life history stages (Key *et al*., 2003; Wirth *et al*., 2004; DeLorenzo *et al*., 2006) to evaluate potential impacts of insecticides and pesticides like permethrin, atrazine, imidacloprid and fipronil in the ecosystem. Additionally, researchers have stressed the importance of *Palaemonetes* species as biomonitoring indicator of human impacts on estuarine and coastal environments (Key *et al*., 2006).

The grass shrimp has also been used as a sentinel species for biomonitoring. The NOAA National Center for Coastal Ocean Science recently published a review discussing the importance of grass shrimp (*Palaemonetes* species) as biomonitoring indicator of coastal condition in estuarine and coastal environments (Key *et al*., 2006).

11.3 ACUTE TOXICITY TESTS

Uyaguari *et al*. (2008) found that Oxytetracycline toxicity to *Palaemonetes pugio* is very low with an LC_{50} of 683.3 mg/L OTC (95% C.I.= 610.8-764.4 mg/L OTC). This confirms the criterion described by Alderman and Hastings (1998) who have explained the low acute toxicity of OTC to aquatic animals. Information about other antibiotic toxicity tests in grass shrimp is not available.

Ferreira *et al*. (2007) conducted 48-h toxicity tests of *Artemia parthenogenetica* exposed to OTC. An LC_{50} of 806 mg/L OTC (95% CI= 650.71-1129.81 mg/L) for nauplii was similar to the values found in this research. Furthermore, the NOEC and LOEC determined by these authors had values as high as our findings: 500 mg/L and 750 mg/L OTC, respectively. These slight differences observed in the LC_{50} and NOEC and

LOEC values are likely due to species differences between the two studies. Adult grass shrimp were generally less sensitive to OTC than other crustaceans. Differences among test species like *Daphnia magna* and *Artemia* for oxytetracycline sensitivity have previously been reported (Okamura *et al*., 2000; Wollenberger *et al*., 2000; Guerra, 2001).

In other studies of OTC effects on crustacean species, Baticados *et al*. (1990) and Williams *et al*. (1992) reported tests of 5 penaeid shrimp larvae stages (*Penaeus monodon* and *P. vannamei*) exposed to oxytetracycline. These authors reported overall LC_{50} levels for all 5 shrimp larval stages ranging from 100-238.4 mg/L OTC.

In summary, results have demonstrated the low toxicity potential of oxytetracycline to adult grass shrimp (Uyaguari *et al*., 2008). Levels as high as 500 mg/L OTC were tolerated by the organisms without causing mortality, but grass shrimp motility was limited at this point. Consequently, these results indicate that concentrations in the field required to cause mortality to *Palaemonetes pugio* would occur only under extraordinary conditions in the environment based upon current biomonitoring results. Levels between 340-1340 ng/L of OTC have been determined in surface water in freshwater streams across the U.S. (Kolpin *et al*., 2002; Schwab *et al*., 2005). Acute OTC effect levels in grass shrimp were more than 500,000 times the maximum surface water concentration of OTC measured in the U. S. OTC levels in sewage effluents between 1290-4600 ng/L have been reported (McQuillan *et al*., 2002; Karthikeyan and Meyer, 2006). Observed acute grass shrimp effect levels were 108,696 times the maximum sewage effluent levels reported. This generally indicates OTC would rarely cause acute effects in grass shrimp. Concentrations up to 475 ppm have been previously reported in sediments beneath salmon production cages and concentrations ranging from 0.1 to 10.2 ppm have been reported in benthonic non-target organisms in the environment (Samuelsen *et al*., 1993; Le Bris *et al*., 1995; Capone *et al*., 1996; Pouliquen *et al*., 1996; Coyne *et al*., 1997; Campbell *et al*., 2001; Montoya and Uyaguari, 2003; Le Bris and Pouliquen, 2004; Reed *et al*., 2004; Ueno *et al*., 2004; Reed *et al*., 2006; Rigos *et al*., 2006; Ferreira *et al*., 2007). A recent study in China has reported concentrations of OTC up to 712 μg/L and 262 mg/Kg OTC in surface water and sediments, respectively (Li *et al*., 2008), indicating that antibiotic resistance of bacteria could be induced and maintained due to levels of OTC present in the environment. Figure 11.2 summarizes OTC concentrations reported in these studies.

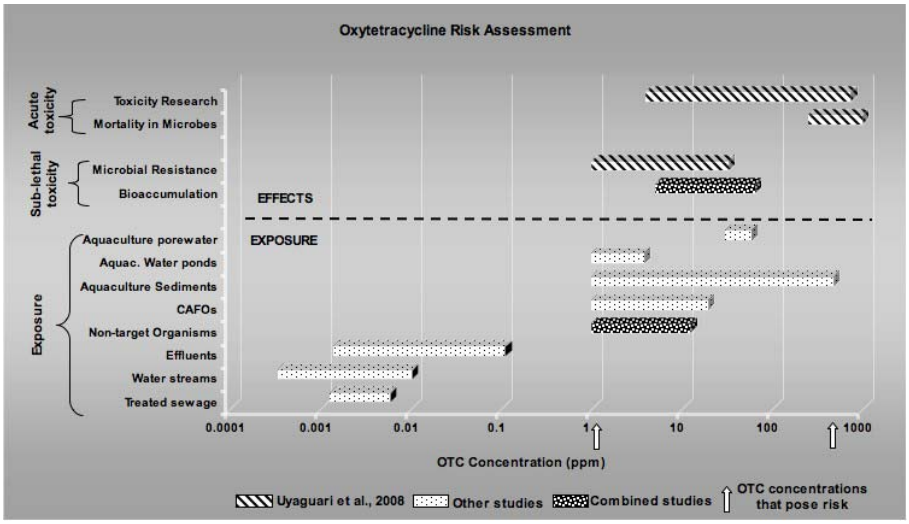

Figure 11.2: Presumptive Environmental Risk Assessment for OTC based upon Results Obtained Uyaguari et al.(2008) as well as Other Published Studies on OTC Effects (Samuelsen, 1993; Doi and Stoskopf, 2000; Li et al., 2008).

11.4 OXYTETRACYCLINE IMPACT ON MICROFLORA

Given the general lack of acute toxicity effects of OTC as described previously, Uyaguari *et al*. (2008) focused on chronic effects on bacterial populations in the grass shrimp digestive tract, as an approach to further evaluate OTC effects. The experimental design in this study used 2 chronic tests based upon microbial population exposure. The first experiment employed concentrations as high as the LOEC (750 mg/L OTC) measured in the acute grass shrimp toxicity tests. Results indicated that bacteria populations were not consistent over time, and after 48h of exposure, all bacteria were killed by the OTC exposure. Similar findings using OTC high doses have been reported from previous studies conducted by Samuelsen *et al*. (1992) and Hansen *et al*. (1992), who evaluated bacteria population response to OTC levels of 300 to 490 ug/g wet sediment under salmon cages; after 2 days of exposure they found significant decreases of 50-70% of the microbial population. This suggests that levels at or above the acute NOEC concentrations for grass shrimp, a significant effect on digestive tract bacteria survival in the grass shrimp would be observed.

Results of a second sub-lethal test at lower OTC concentrations (1, 16 and 32 mg/L OTC) did not show significant bacterial mortality; however, more demonstrable effects on antibiotic resistance were observed. There

were no marked differences in total bacterial population densities for shrimp exposed to 1, 16 and 32 mg/L OTC and control. Average levels of $3.1x10^4$ cfu/g from shrimp were measured in our experiment. Significant population changes in terms of bacterial abundance have been reported for penaeid shrimps exposed to pharmaceuticals in ponds (Chang *et al*., 1996; Sung and Hong, 1997; Sung *et al*., 1999). Nevertheless, these differences in bacterial abundance may be attributed to seasonal changes and abiotic factors present in the pond where animals are cultivated than therapeutic agent effects *per se* (Tendencia and de la Peña, 2001).

The predominance of *Aeromonas* and *Vibrio* species from the digestive tract in other species of shrimp and water isolates related to environmental and aquatic sources have previously been characterized and reported (MacDonell *et al*., 1982; Spanggaard *et al*., 1993; Pursell *et al*., 1996; Sung *et al*., 1999; Sung *et al*., 2001). Phenotypic characterization tests enabled to classify the microbial species present in the shrimp homogenates. In general, the microbial community in the shrimp digestive tract during 96h exposure was dominated by *V. alginolyticus* (25.9%), *Aeromonas hydrophila* (18.8%), *Vibrio* spp. (17.7%) and *Vibrio vulnificus* (13.9%). These findings correlate well with the results of previous studies and demonstrate the accuracy of the API analytical profile index as a tool to identify 99.4% of bacterial isolates commonly found in aquatic and marine microbiological samples (MacDonell *et al*., 1982).

DePaola *et al*. (1995) conducted studies to analyze and determine bacterial diversity and resistance levels in catfish intestinal contents and pond water samples. These authors did not find any prevalent trend among the bacteria associated with OTC treatment used for the ponds. Similar results were obtained by Kerry *et al*. (1997) when OTC was administered to Atlantic salmon. In our studies, the bacterial composition (genus and species) of the digestive tract was compared to evaluate differences over time and dose. Uyaguari *et al*. (2008) performed a cluster analysis of grass shrimp microbial community where they showed as exposure time to OTC increased, there was an effect of time and OTC concentrations to reduce the growth of *Vibrio vulnificus*, *V. parahaemolyticus*, *V. cholerae*, and *Vibrio* spp. when compared to the control group; while a clear trend of increased growth over time was observed for *Vibrio alginolyticus*. This suggests that *Vibrio alginolyticus* is more resistant to OTC exposure and that other Vibrios (*V. cholerae*, *V. parahameolyticus*, *V. vulnificus* and other *Vibrio* spp.) are less resistant.

The bacterial composition interactions and changes over time among bacteria groups in shrimp have been well documented (Gomez-Gil *et al*.,

1998; Rengpipat *et al*., 1998; Gatesoupe, 1999; Gomez-Gil *et al*., 2000; Rengpipat *et al*., 2000; Gullian *et al*., 2004), using *Vibrio alginolyticus* as a probiotic bacteria, which was the dominant species observed in this study. Moreover, microbial population changes affected by OTC occurrence in animals, water and sediment were discussed by Angka (1997), Inglis *et al*. (1997) and Anderson and Levin (1999). Uyaguari *et al*. (2008) reported that there is a greater OTC dose effect than a temporal effect on the microbial community. They also observed a rapid decrease in species diversity as well as numbers at higher OTC concentrations.

11.5 OXYTETRACYCLINE RISK ASSESSMENT

The potential impacts to aquatic life are based on two concepts: the predicted environmental concentration (PEC) and the predicted no observable effect concentration (PNOEC). In general, if the ratio PEC/PNOEC is less than one, the environmental risk is deemed acceptable (Cunningham *et al*., 2006). Ratios greater than one indicate clear risk. This research has tested the grass shrimp ability to act as a bio-monitor for antibiotic resistance in an estuarine environment microcosm. Moreover, the study utilized a more sensitive approach to estimating environmental risk related to the use of the antibiotic oxytetracycline by assessing the impact on the shrimp bacterial microflora.

Very few research studies have been conducted to estimate OTC impact in non-target organisms. Relatively little information is available regarding uptake and bioaccumulation of antibiotics in *P. pugio*. The use of grass shrimp as a bioindicator not only permits testing of the spatial influence of pharmaceuticals in the marine environment, but it could also provide important estimations with respect to seafood safety and water quality.

From an environmental risk assessment perspective oxytetracycline has demonstrated to induce bacterial resistance, even at low environmentally relevant concentrations, has been commonly reported in aquaculture facilities and adjacent ecosystems. A presumptive environmental risk assessment (Figure 11.2) using results obtained in this study and other studies clearly indicates that acute toxicity effects and bioaccumulation in grass shrimp and alterations in the grass shrimp microbial population community and antibiotic resistance within this microbial community would be expected at environmental levels of OTC measure in surface waters, effluents and sediments associated with

aquaculture and combined animal feeding operations but not at levels measured with sewerage treatment plant effluents and surface waters of the U.S. in general. This suggests that OTC exposure from untreated effluent from aquaculture and combined feeding animal operations may pose greater risks than the risks associated than OTC exposure from sewerage treatment plant effluent. This presumptive risk assessment requires additional field monitoring and assessment to confirm risk estimates suggested in this presumptive risk assessment.

The use of grass shrimp digestive tract microbial flora as a biomonitoring tool demonstrated that antibiotics like OTC do affect the digestive tract microflora and may be useful to assess the likely impact and risks of pharmaceutical(s) in the environment. Given the trophic importance of the bacterial microflora in crustaceans, this has the potential to affect feeding/assimilation efficiencies and may potentially affect growth and reproduction. The uncertainty of these physiological effects on feeding/assimilation efficiencies requires additional study but underscores the high degrees of certainty and levels of significance of predicted impacts in current risk assessment models, which do not address or consider antibiotic resistance and alteration in digestive tract microbial flora.

In a previous study conducted by Uyaguari *et al*. (2008), they demonstrated the existence of sub-lethal responses in digestive tract bacterial flora (e.g. *Vibrio* and *Aeromonas* species) which are mainly recognized as causative associated agents of human diseases. This study indicated that pharmaceuticals in the environment may have potential effects to increase microbial resistance for these public health pathogens to both humans as well as aquatic organisms. The transport mechanisms for antimicrobial agents in the environment may require additional analysis and consideration, in particular to determine the potential impact of these agents associated with numerous diseases in marine organisms. It is important to mention that Uyaguari *et al*. (2008) study relied only in culturable-dependent analysis. To the best of our knowledge, only one study to date has used new culture-independent methods of metagenomics to examine the presence of antibiotic resistance genes in nature (Riesenfeld *et al*., 2004). This metagenomic approach does not rely on bacterial culturing or PCR thus these researchers were able to identify several novel antibiotic resistance genes in soil collected at an agricultural site. This suggests that the large reservoirs of uncharacterized antibiotic resistance genes in nature can be determined using a metagenomic approach (R. S. Norman, University of South Carolina, Columbia, *personal communication*). Further research

considering non-culturable dependent analysis would be necessary to analyze the true diversity of novel antibiotic resistant bacteria using non-target organisms.

Future studies should also consider using lower OTC sub-chronic exposures or different *Palaemonetes pugio* life stages as well as other crustacean and vertebrate species. More research is needed to fully measure the environmental impacts on non-target organisms. If the final fate of pharmaceuticals is indeed the sediments, then the use of benthic species like clams, amphipods, isopods, polychaetes and copepods will be required to fully assess the most vulnerable organisms in aquatic environments, particularly where aquaculture facilities are located.

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12

Current Status of Shrimp Farming in India

ARUNACHALAM MANIMEKALAN, SATYANARAYANA MASABATHULA AND DEVARAJAN THANGADURAI

12.1 INTRODUCTION

India, by virtue of its 8,118 km long coastline, 2.02 million sq. km of Exclusive Economic Zone (EEZ) and extensive geographical stretch with varied terrain and climate, supports a wide diversity of inland and coastal wetland habitats. It has been estimated that there are 3.9 million ha of estuaries and 3.5 million ha of brackishwater areas in the country. Out of this, 1.2 million ha of coastal area has been identified as suitable for brackishwater aquaculture and through the use of sustainable practices this resource can yield optimum quantities of shrimp and other commercially valuable fin and shell fish species.

The shrimp farming areas are mainly located in the coastal states of Andhra Pradesh, West Bengal, Kerala, Orissa, Tamil Nadu, Karnataka, Maharashtra, Gujarat and Goa. The major markets for Indian shrimp are Japan, Western Europe and USA. Today, India stands amongst the major shrimp producing countries having a growth rate of about 300 per cent over the last decade. In India, shrimp farming has been traditionally practiced in the coastal states of West Bengal and Kerala. The traditional *trap and culture* system was characterized with low production levels of mixed species of fin and shell fishes. The importance of introducing scientific farming techniques to increase production and productivity from the traditional system was felt and the Indian Council of Agricultural Research (ICAR) implemented an All India Coordinated

Research Project on Brackishwater Fish Farming (1973-1984) to develop and test various farming technologies under different agro-climatic conditions of the country. The main centre of the project was located in West Bengal and the other centres were located in Orissa, Andhra Pradesh, Tamil Nadu, Kerala and Goa for demonstrating the technologies to the small-scale farmers.

Simultaneously, the shrimp hatchery technology was also introduced into the country and two commercial hatcheries were established in the late eighties with initiative from the MPEDA. With the establishment of more hatcheries in the private sector, the country witnessed a faster development of shrimp farming during 1990 to 1994. The culture practice was also gradually intensified and varied levels of intensification were noticed depending on the investment capabilities of the farmer/ entrepreneur. Stocking densities of 2 to 30 No/m^2 were used under different systems by the farmers.

Like any other agriculture/ animal husbandry practice, shrimp culture was also affected by health and disease problems. Initially, some of the bacterial diseases were noticed which were more or less localized and the mortality levels were not very high. Later in 1995, viral diseases such as *monodon baculo virus* and *white spot virus disease* affected the farmed shrimp and there was a slump in shrimp farming. Reasons such as heavy stocking densities and poor farm management practices were attributed to the shrimp diseases out break in the country. Following the verdict of the Supreme Court of India and the establishment of Aquaculture Authority with powers to issue licenses and guidelines, the shrimp culture sector is gradually going through a regulated regime and is slowly returning to its normal production level.

Aquaculture in India has a long history, there are references to fish culture in Kautilya's *Arthashastra* (321-300 B.C.) and King Someswara's *Manasoltara* (1127 A.D.). The traditional practice of fish culture in small ponds in eastern India is known to have existed for hundreds of years; significant advances were made in the state of West Bengal in the early nineteenth century with the controlled breeding of carp in *bundhs* (tanks or impoundments where river conditions are simulated). Fish culture received notable attention in Tamil Nadu (formerly the state of Madras) as early as 1911, subsequently, states such as Punjab, Uttar Pradesh, Baroda, Mysore and Hyderabad initiated fish culture through the establishment of Fisheries Departments.

Aquaculture has developed rapidly over the last three decades to become an important economic activity world-wide. It has confronted many of the developmental problems in this relatively short period

including sector competition, over production, trade restrictions, overcapitalization and concerns over environmental impacts. The significance of aquaculture in the context of global food production sector, the management of aquatic resources and the socio-economic development of coastal rural areas is now fully appreciated world-wide. Significant advances have also been made globally to make shrimp aquaculture development responsible and sustainable.

12.2 SHRIMP AQUACULTURE - GLOBAL AND INDIAN SCENARIO

Shrimp farming is relatively new and its introduction on a commercial scale can be traced back to the early seventies. Despite its relative newness, one-third of world shrimp production is now from farmed shrimp. During the period from 1984 to 1997, 63 countries were listed in FAO (1999) aquaculture statistics as having produced shrimp at one time or another. In 1984, only 29 countries had reported any shrimp production. The shrimp producing countries are found in all regions, including Europe and the Middle East.

Global fisheries production reached 130.2 million tonnes in 2001, having doubled over the last thirty years (FAO, 2001). However, a significant part of the increase has come from aquaculture. While output from capture fisheries grew at annual average rate of 1.2 percent, output from aquaculture (excluding aquatic plants) grew at a rate of 9.1 per cent reaching 39.8 million tonnes in 2002. This rate is also higher than for other animal food producing systems such as terrestrial farmed meat (FAO, 2003). Much of this aquaculture expansion has been due to China whose reported output growth far exceeded the global average. However, if excluded, world aquaculture output growth during the last thirty years was more moderate with declining rates of expansion (FISHSTAT+, 2004).

The following account details some of the salient features of shrimp aquaculture development in the major shrimp producing countries. Thailand has been the leading world producer of farmed shrimp from 1993 onwards. About 80.0% of the shrimp farms are owned by small-scale farmers, operating 1-2 ponds, each ranging in size from 0.16-1.6 ha. The Government of Thailand provides considerable assistance to these farmers by developing sea water irrigation system (SIS) which brings in clean sea water that individual farms can tap. The SIS incorporates pre and post water treatment measures and a good system of shrimp disease

diagnosis and prevention is made available to the shrimp farmers. Presently, extensive, semi-intensive and intensive types of farming systems are practiced in the country. Thailand has also demonstrated some of the most sustainable shrimp farming methodologies and the shrimp farms under Kung Krabaen Bay Royal Development Study Centre in Chanthaburi Province are excellent examples for replication (Aquaculture Authority of India, 2001).

Indonesia is the second largest shrimp producing country after Thailand. In the province of Lampung which is located in southern Sumatra, one of the world's largest shrimp farm under the control and management of one company can be found. P.T. Dipasena Citra Darmaja has established 18,000 individual plastic-lined culture ponds covering about 4,500 ha in a 16,000 ha site. It has also obtained the rights to further develop at least 50,000 ha of land. Most of the ponds measure 2,500 m^2 gross area, with a few measuring half a hectare. As provided for under Indonesian law, any aquaculture development over 30 ha in Java and 50 ha in islands outside Java has to be developed under the Tambak Inti Rakyat (TIR) or nucleus-estate concept. Indonesia still has large undeveloped land in the outer islands, particularly in Sumatra and therefore has the potential to become the world's largest farmed shrimp producer, if the farming practices are done in a sustainable and responsible manner (Aquaculture Authority of India, 2001).

China originally started with freshwater aquaculture but has now moved to coastal salt water aquaculture in a big way. Without the shrimp farms the coastal flat lands were useless for anything else. The meteoric rise in the Chinese shrimp farming was made possible by a massive development programme along the Bohai Bay coastline in the north east all the way down to Fujian in the south. Every year, thousands of hectares were developed by the Chinese government when it came to the realization that shrimp farming was the best way of productive utilization of vast tracts of arid saline-alkaline coastal flat lands, providing employment and earning foreign exchange. In 1993, China found that overstocking and lack of provision for treating wastewater discharge resulted in decline in production. However, mitigation measures were soon adopted and presently extensive, semi-intensive and intensive farming methods are practiced for shrimp farming (Aquaculture Authority of India, 2001).

Unlike other South-East Asian countries, Philippines lacks the abundance of resources suitable for shrimp aquaculture. At its peak in 1993, Philippines produced 95,816 metric tonnes of all species, but mostly of the giant tiger shrimp. The total production in 1997 fell largely

due to shrimp disease. However, shrimp growers in Negros Occidental which used to be the centre of intensive shrimp farming are slowly trying out lower densities, using pro-biotics, pond bio-remediation techniques and even mixed culture with fish species such as *Tilapia*. The South East Asian Fisheries Development Centre (SEAFDEC), an inter-governmental organization with headquarters in Bangkok is providing technical back-up to shrimp farmers in the Philippines for making farming practices more sustainable (Aquaculture Authority of India, 2001).

The culture systems used are extensive, semi-intensive and intensive. The middle East, is also striving to become a shrimp producing region. Many of the countries in the region including United Arab Emeritus, Kuwait and Yemen have initiated moves to venture into shrimp farming. So far, large-scale developments have taken place only in two countries, Saudi Arabia along the Red Sea and Islamic Republic of Iran along the Persian Gulf. Iran is moving very fast after culture trials under a UNDP/FAO Project showed that it is possible to breed and grow local species found in the Persian Gulf which has an average salinity of 38 ppt. Initially two species were being farmed - *P. semisulcatus* and *P. indicus*. However, due to slow growth of the former when farmers shifted to a locally milled feed, all the farms are now stocked with *P. indicus*. In the year 2000, shrimp farming expanded even faster than it did in 1999. Over 12,000 ha of new farms are being designed and built in the coastal areas. Presently, the harvest average 1.8 metric tonnes per hectare and almost the entire crop is exported to Europe.

Ecuador, the only country outside Asia, stands fourth position in production. As early as 1984 it was already producing more than any of the major shrimp producing countries in Asia. The production touched a peak of about 1,30,000 metric tonnes in 1997. Extensive and semi-intensive methods of culture are practiced in Ecuador. Practically all the Latin American countries from Mexico to Peru produce shrimp. However, except for Mexico, Honduras and Columbia which in 1997 produced 16,000, 12,000 and 10,000 metric tonnes respectively, all the rest produced less than 10,000 metric tonnes (Aquaculture Authority of India, 2001).

12.3 DEVELOPMENT OF SHRIMP AQUACULTURE IN INDIA

India, by virtue of its 8,118 km long coastline including coasts of islands (WWF, 1992), 2.02 million sq. km of Exclusive Economic Zone (EEZ) and

extensive geographical stretch with varied terrain and climate, supports a wide diversity of inland and coastal wetland habitats. It has been estimated that there is 3.9 million ha of estuaries and 3.5 million ha of brackishwater areas in the country (WWF, 1992). Out of this, 1.2 million ha of coastal area has been identified as suitable for brackishwater aquaculture and through the use of sustainable practices this resource can yield optimum quantities of shrimp and other commercially valuable fin and shell fish species.

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Simultaneously, the shrimp hatchery technology was also introduced into the country and two commercial hatcheries were established in the late eighties with the initiative from the Marine Products Export Development Authority (MPEDA). With the establishment of more hatcheries in the private sector, the country witnessed a faster development of shrimp farming between 1990-1994. The culture practice was also gradually intensified and varied levels of intensification were noticed depending on the investment capabilities of the farmer/ entrepreneur. Stocking densities of 2 to 30 nos/m^2 were used under different systems by the farmers. Like any other agriculture/animal husbandry practice, shrimp culture was also affected by health and disease problems. Initially, some of the bacterial diseases were noticed which were more or less localized and the mortality levels were not very high. Later in 1995, viral diseases such as *Monodon baculo* virus and white spot virus disease affected the farmed shrimp and there was a

slump in shrimp farming. Reasons such as heavy stocking densities and poor farm management practices were attributed to the shrimp diseases out break in the country. Following the verdict of the Hon'ble Supreme Court of India and the establishment of Aquaculture Authority with powers to issue licenses and guidelines, the shrimp culture sector is gradually going through a regulated regime and is slowly returning to its normal production level (Aquaculture Authority of India, 2001).

12.3.1 Status of Shrimp Aquaculture in the Coastal States and UTs

Starting in a small way in the late 1980s, commercial shrimp aquaculture became a significant activity in the early 1990s in selected pockets along the country's vast coastline. The states with the highest concentration of shrimp culture farms are Andhra Pradesh, Tamil Nadu and Orissa. Some of the west-coast states like Maharashtra and Goa also developed commercial shrimp farms, but the overall area is much less as compared to the states on the east coast. The area and production under traditional systems was stagnant for decades as there was no effort made for optimization of the production through adoption of scientific methodologies.

Commercial shrimp farming developed on account of the government's policy to promote shrimp culture in view of its potential to utilize the vast saline tracts along the coastline, provide employment opportunities to coastal rural population and to earn valuable foreign exchange. After the liberalization of Indian economy in 1991, development of shrimp culture gained greater momentum. Individual entrepreneurs were also encouraged to take up shrimp farming with both financial and technical support. The opposition to shrimp culture has stemmed from both socio-economic and environmental issues. The socio-economic issues include land alienation, displacement of coastal communities from open access to public lands used by them for fish and net drying, grazing etc., The environmental issues include Stalinization of groundwater and productive agricultural lands, contamination of ground and surface waters with organic wastes of shrimp farming, destruction of mangroves, loss of mud-flats which result in changes in conditions.

12.3.1.1 Shrimp hatcheries

Success of any farming activity is dependent on the availability of quality seed in required quantity. As the demand for shrimp seed increased with the development of commercial shrimp farming, a number of commercial

shrimp hatcheries have also been established. Most of these hatcheries have state of the art facilities for producing healthy and disease free post-larvae. The details of the state-wise distribution of shrimp hatcheries are presented in Table 12.1.

Table 12.1: State wise shrimp hatcheries and annual larval production

State	Number of shrimp hatcheries	Annual production level (in millions)
West Bengal	3	-
Orissa	9	445
Andhra Pradesh	133	6,909
Tamil Nadu	72	2,933
Kerala	24	248
Karnataka	12	240
Maharashtra	6	51
Gujarat	1	5
Total	260	10,831

Presently, there are 260 shrimp hatcheries in operation in the country with a total annual production capacity of 10.8 billion shrimp seed (PL20). These hatcheries are mostly located in the east-coast states, with the maximum number (133) in Andhra Pradesh followed by 72 in Tamil Nadu. Location of the large number of hatcheries on the east coast is also because of the greater availability of brood shrimp in the Bay of Bengal as compared to the Arabian sea.

12.3.1.2 Feed mills

Shrimp feed production is one of the allied activity which is associated with the sustainable development of shrimp farming in the country. At present there are 33 shrimp feed mills with a total installed capacity of 1,50,000 metric tonnes (Table 12.2) Andhra Pradesh with 24 mills and Tamil Nadu with three mills together contribute to about 87.0 per cent of the total feed production capacity.

Table 12.2: State-wise details of shrimp feed production in India

State	No. of feed mills	Installed capacity (MT)
Kerala	2	12,000
Karnataka	3	1,000
West Bengal	1	6,000
Andhra Pradesh	24	1,10,500

Tamil Nadu	3	20,500
Total	33	1,50,000

12.3.1.3 Post-harvest infrastructure facilities and exporters

The development of shrimp farming and the increased production from aquaculture has led to an increase in the quantum of exports. To cope with the increased load and meet the requirements of the importing countries and the growing domestic market on product quality, there has been a general increase in the post-harvest infrastructure for the seafood processing and exports. The details of the infrastructure facilities as on 31.3.1990 and 31.3.2000 are given in Table 12.3.

Table 12.3: Details of infrastructure facilities available for shrimp processing

Category	Number (as on 31.3.1990)	Capacity (t)	Number (as on 31.3.2000)	Capacity (t)
Freezing plant	231	2,296	394	8,439
Ice plant	132	1,854	157	2,970
Cold storage	304	42,458	479	1,05,991
Exporters	864	-	1,549	-

12.3.2 Shrimp Farming and Production in Gujarat

Gujarat with 3,76,000 ha of potential brackishwater land, occupies the second place among the maritime states next to West Bengal. However, only 997 ha have been developed for farming till 1999-'98 (Government of Gujarat, 2001). During the year 1999-2000 the area of shrimp farming declined to 447 ha. In 2000-01, the area of shrimp farming increased to 2,377 ha and now only 1,298 ha of land is under shrimp farming (Table 12.4).

Table 12.4: Details of area under shrimp farming and annual shrimp production in Gujarat

Year	Area (ha)	Annual production (t)	Average shrimp production (t/ha)
91-92	231	170	0.74
92-93	360	200	0.56
93-94	475	500	1.05
94-95	700	700	1.00
95-96	804	546	0.68

96-97	997	572	0.57
97-98	997	235	0.24
98-99	315	256	0.81
99-00	447	340	0.76
00-01	2,377	668	0.28
01-02	2,480	750	0.30
02-03	1,240	1,130	0.91
03-04	1,013	1,510	1.49
04-05	891	1,500	1.68
05-06	1,297	3,322	2.56

The annual shrimp production was only 170 tonnes during 1991–92 and it has increased to 700 tonnes during the year 1994–95 and started falling to 340 tonnes during 1999–2000 and now it increased to 3,322 tonnes. The average annual production per hectare has been very poor (0.24 t/ha) during 1997–98 and now it increased to 2.56 t/ha is the highest production.

In Gujarat, the average area under shrimp production has been 974.93 ha with a total production of 826.6 tonnes with an average about 1 tonne per ha. The area under production and the total production have been in the parallel lines. It implies that an increase in the area has been followed by increase in the total production and hence the correlation values have been positive and significant.

Thus Gujarat has performed well in shrimp production, but when the average output per ha was considered, the relationship between the average productions namely productivity and the area under shrimp production had been negative to a tune of -0.156. That is to say productivity has gone down with increase in the area which may be due to various ecological, social, cultural and environmental factors. This fact has to be taken care of in harnessing the productivity per unit area in the state.

12.3.3 Shrimp Farming and Production in Maharashtra

Maharashtra occupies fourth place among the maritime states with about 80,000 ha of potential brackishwater area for shrimp farming. Out of this, only about 2,400 ha have been developed for shrimp farming till 1994-95. However, in 1998-99, the total area under shrimp farming got reduced to 426 ha (Table 12.5).

Table 12.5: Details of area under shrimp farming and annual shrimp production in Maharashtra

Year	Area (ha)	Annual production (t)	Average shrimp production (t/ha)
91-92	1,059	930	0.88
92-93	1,900	1,050	0.55
93-94	2,100	300	0.14
94-95	2,400	400	0.17
95-96	2,164	2,400	1.11
96-97	929	523	0.56
97-98	970	700	0.72
98-99	426	409	0.96
99-00	533	390	0.73
00-01	1,114	385	0.35
01-02	4,450	460	0.10
02-03	4,480	930	0.21
03-04	615	981	1.60
04-05	524	1,068	2.04
05-06	647	683	1.06

During the year 2002-03 the area of shrimp production increased to 4,480 ha and now the area under shrimp production is only 647 ha. The annual total production of the state is not constant and keeps varying from year to year. The annual total production during 1992-93 is 1,050 tonnes but the production fell to 300 tonnes in the following year. The state has recorded highest shrimp production of 2,400 tonnes during 1995-96. The present annual shrimp production is 683 tonnes. The average production levels during 2001-02 have touched a very low level of 103 kg/ha and the highest average production reached 2,038 kg/ha during 2004-05.

In Maharashtra, average area under shrimp production had been 1620.7 ha with an annual production of 0.74 tonnes/ha. The relationship between the area under shrimp production and the total shrimp production has been positive meaning the increase in the area has been followed by an increasing production also.

This is a welcome trend, but when the average production per hectare and the area under production was considered, the relationship had been negative and significant. This implies that in Maharashtra, the productivity has been negatively related with the total area under shrimp

production. It may imply that the management of production has not been scientific and economical which needs to be seriously considered if the profit is to be harvested.

As per the information provided by the Government of Maharashtra, about 90 farms with 712.4 ha area are presently under shrimp farming. All these farms are creek-based and 66 farms are within costal regulation zone and the remaining 24 farms are outside CRZ. Farm-wise area coverage has been estimated as farms with <2.0 ha - 5 per cent; 2-5 ha - 20 per cent; 5 - 40 ha 31 per cent; and >40 ha - 44 per cent (Government of Maharashtra, 2001).

12.3.4 Shrimp Farming and Production in Goa

In Goa state, a total of 18,500 ha of potential brackishwater area are available for shrimp farming. Around 525 ha of brackishwater area is under traditional farming during the year 1991-92. During the year 2003-04 it has increased to 963 ha but during 2005–06 the shrimp farming is practiced only in 331 ha (Table 12.6)

Table 12.6: Details of area under shrimp farming and annual shrimp production in Goa

Year	Area (ha)	Annual production (t)	Average shrimp production (t/ha)
91-92	525	300	0.571
92-93	550	350	0.636
93-94	575	400	0.696
94-95	600	450	0.750
95-96	650	550	0.846
96-97	650	580	0.892
97-98	650	590	0.908
98-99	600	590	0.983
99-00	770	840	1.091
00-01	929	966	1.040
01-02	930	1,200	1.290
02-03	930	710	0.763
03-04	963	700	0.727
04-05	295	534	1.810
05-06	331	659	1.991

The annual shrimp production was 300 tonnes during 1991-92 which reached maximum of 1,200 tonnes during 2001-02. However, the present shrimp production is around 660 tonnes. The average shrimp production

was 0.571 t/ha during 1991-92 and the highest yield of 1.810 t/ha was reached during 2004-05.

In Goa, the average area under shrimp production and the total shrimp production in the state are more or less positively related. This implies that the shrimp production in the state has been hand in hand with the area under shrimp production. The correlation value also reveals that the above variables have been positively related with a correlation coefficient of 0.644 being significant of 1 per cent level.

Statistically, the above two variables are linked positively, but when the average shrimp production per hector is related to the total area under shrimp production, the correlation value has been negative being -0.437 which is significant. That is to say the productivity is negatively related to the area under production. This again exposes that the production of shrimp per unit area has not been carried out scientifically and economically and to the minimum level of profit expectation against a set of risks and uncertainties.

Shrimp farming in Khazan lands is generally practiced during December to April after the paddy harvest. However, development of improved traditional farming in the state is very slow with only 110 ha area comprising 48 farms being developed so far. It is estimated that 33 per cent of the total farm area comprises farms under 2.0 ha size; 33 per cent within 2.0-5.0 ha and 34 per cent above 5.0 ha. All the 48 farms are creek-based with 28 farms within CRZ and 20 farms outside CRZ (Government of Goa, 2001).

12.3.5 Shrimp Production in Karnataka State

A total of 8,000 ha of potential brackishwater area is available in the state. In Karnataka, traditional shrimp farming was carried out in 2,542 ha during 1991-92 and it reached the highest of 3,635 ha in 1999-2000. At present 3,262 ha of land is under shrimp cultivation. Presently, about 488 ha area is developed in the state under the improved traditional system of shrimp culture.

During the year 1991-‘92 the annual production was 1,100 tonnes in 2,542 ha with an average of 433 kg/ha and the maximum shrimp production was 3,600 tonnes in 3,230 ha during 2001-02. The average production is about 1,115 kg/ha, and the total cultivated shrimp production has fallen to 1,843 tonnes with an average of 565 kg/ha.

In Karnataka, the analysis revealed an entirely divergent view i.e. shrimp farming has been totally a profitable enterprise in this state. The area and shrimp production are positively related with very strong

correlation r value of 0.596, and it is significant. It can be inferred that in Karnataka, shrimp farming is being practiced profitably with an amount of acceptable motivation. From the analysis, it can be seen that Karnataka state is the role model in maximizing the profit production and also the productivity for other areas to emulate. It is estimated that 36 per cent of the total area is under farms of less than 2.0 ha size; 31 per cent in farms between 2-5 ha and 33 per cent in farms above 5.0 ha size (Government of Karnataka, 2004). The average production levels in the state is about 700 kg/ha.

12.3.6 Shrimp Farming and Total Production in Kerala

The state has a sprawling brackishwater area of nearly 65,000 ha suitable for shrimp farming. Out of this about 14,500 ha has been utilized for shrimp aquaculture. Major portion of this area is under traditional prawn filtration fields, locally known as `Chemmeen kettu'. This culture operation is an age-old practice wherein shrimp culture and paddy cultivation is practiced in rotation during summer and monsoon seasons respectively. Selective/supplemental stocking is also practiced in traditional culture systems with a view to increasing the production levels of the desirable species.

The total shrimp production of Kerala in 1991-92 was 9,500 tonnes in over 13,145 ha of land with an average of 723 kg/ha (Government of Kerala, 2001). During the year the annual production increased to 12,000 tonnes, but subsequently, the annual production gradually decreased to 5,740 tonnes in the year 2001-02 and the present production is about 6,883 tonnes in 2005-06. The highest production of 830 kg/ha was recorded during 1993-94 and the lowest was 371 kg/ha in 2001-02.

In Kerala, the area under shrimp production is large when compared to other states. But the total production has been remarkably lower to a tune of 8,277.87 tonnes i.e. the production has not been commensurate with the area. The total area under production and the total shrimp production are negatively related and also the average production per hectare with the area is negatively related with high intensity. This is to say that the shrimp farming has not been to the minimum expected level of economy. It implies that the shrimp farmers in the state are to be trained and motivated to make shrimp farming a profitable enterprise. As per the estimates provided by the government of Kerala, 2,166 ha is presently under improved traditional system. Of this area, 36 per cent is

under 2.0 ha; 18 per cent is under 2.0-5.0 ha and 46 per cent is under farms with area more than 5.0 ha.

12.3.7 Shrimp Production in Tamil Nadu

Tamil Nadu with a total of 56,000 ha of potential shrimp farming area ranks sixth among the maritime states of the country. Although a total of 4,455 ha has been developed (Government of Tamil Nadu, 2001) in the state for shrimp farming, yet only a maximum of 2,879 ha has been reported in use for shrimp farming (MPEDA, 2000). The area under shrimp production was only 400 ha in 1991-92 which increased to 2,079 ha during 1995-96. However, it declined to 670 ha during 1997-98. However, it again showed an upward increase and reached 4,916 ha during 2005-06 (MPEDA, 2004).

In 1991-92 the total shrimp production was only 700 tonnes, but it increased to 3,000 tonnes during 1994-95. In 1995-96 the total production has fallen to 1,092 tonnes, which declined further to 400 kg/ha during 1995-96 due to outbreak of viral diseases. The average annual shrimp production varied between 1,500-1,600 kg/ha and the highest average production of 2,075 kg/ha was reported during 1992-93.

In Tamil Nadu, the area under shrimp production and the total shrimp production have been more or less positively related. The relationship between the total area and the total production has been the highest when considered to other States. The r value is 0.937. But the productivity aspect is again being negatively related to the total area which implies that it is not economically viable. Of the total area developed in Tamil Nadu, farms covering 3178 ha are creek-based and the remaining 1,277 ha are sea-based. About 3,268 ha of the developed area are within the CRZ while 1,187 ha are outside CRZ (Government of Tamil Nadu, 2001).

12.3.8 Shrimp Farming and Shrimp Production in Puduchery

The total fish production of Puduchery for 1998-99 was 42,700 tonnes. Of this, 38,600 (84.31%) tonnes was from marine sector alone. The total annual shrimp production ranges between 10 to 37 tonnes. About 155 ha of shrimp farms have been developed from the available potential area of 800 ha. Of this area, 92 per cent are creek-based farms. All the farms have been developed outside the CRZ (Government of Puduchery, 2001).

It is further reported that presently about 15-20 per cent of the total farm area only is under operation (MPEDA, 2000).

In Puduchery, the area under shrimp production on an average has been 29.5 ha producing an yield of 22.6 tonnes. The productivity per ha has been 0.99 tonnes or to say 1 tonne/ha which is remarkable. Surprisingly, in Puduchery, the relationship between the area and total production and also per hector production meaning the productivity has been negative. The production and the area under production are negatively related with significant value of the correlation coefficient of 0.118. Therefore, it could be implied that in Puduchery, shrimp production has nothing to do with area but it may have been due to other factors responsible for increase in shrimp production. Further 15 per cent of the farms are small with less than 2.0 ha farm holding; 41 per cent within 2.0 - 5.0 ha holding; and 44 per cent having holdings larger than 5.0 ha.

12.3.9 Shrimp Farming and Production in Andhra Pradesh

Andhra Pradesh is the largest maritime state of India and contributed 86,210 tonnes of prawn during the year 2002-'03 of which marine fish production was estimated at 2,12,000 tonnes. In 1990, a total of 6,000 ha was under shrimp farming which increased to 1,01,940 ha during 2001-'02. However, now the shrimp farming area got reduced to almost half and the present total farming area is about 57,712 ha.

During 1991-92, the annual production was 9,500 tonnes from 6,000 ha with the maximum average production of 1,583 kg/ha. The State recorded the maximum shrimp production of 86,210 tonnes from 93,000 ha during 2002-03. At present the total cultivated shrimp production is about 70,669 with an average of 1.225 kg/ha. Out of 78,702 ha, about 75,625 ha (96%) is based on brackishwater/ estuarine creeks and 3,077 ha (4%) is based on sea. It is estimated that about 79 per cent of the creek-based farms and 40 per cent of the sea-based farms are located beyond coastal regulation zone. The average rate of production was above 1000 kg/ha till 1994-95, but dropped to about 551 kg/ha during 1999-2000. During 2000–01, the annual production again started rising from 745 kg/ha and reached 1,224 kg/ha during 2005-06.

In Andhra Pradesh, the area as well as the production of shrimp is large when compared to other states. But an analysis of the area under shrimp production, the total shrimp production and also the productivity per hector shows that the total production and the total area under shrimp production are positively significantly correlated with a

correlation coefficient of 0.834 with area and the total production. It can be concluded that the total production has a remarkable relationship with the area under shrimp production. Though, Andhra Pradesh is the leading state, the relationship between the productivity and the total area turned to be negative with r value being -0.655 being significant at 1 per cent level. This implies that Andhra Pradesh also lacks in all other skills required to increase the shrimp production. This particular aspect is to be seriously taken care of in making shrimp farming a most profitable enterprise in the years to come. Further 75 per cent of the total area is owned by small farmers with less than 2.0 ha farm holding (59,175 ha); 8 per cent of the area is in farm holdings of 2.0-5.0 ha (5,811 ha); 17 per cent in farms of larger than 5.0 ha (Government of Andhra Pradesh, 2001).

12.3.10 Shrimp Production in Orissa

In Orissa, about 11,332 ha have been developed for shrimp farming by 1996-97. But in 1998-99 only 8,000 ha were in operation. As per the Government of Orissa, a total of 12,627 ha of farms are presently in operation in the State (Government of Orissa, 2001). About 45 per cent of the shrimp farming area is located on Chilka periphery. The remaining 55 per cent are creek-based and distributed over the 34 different estuary/ creek systems of the state. During 1991-92 the annual production was 3,800 tonnes from 7,417 ha with an average of 512 kg/ha and the state recorded the maximum shrimp production of 12,390 tonnes from 12,116 ha during 2003-04 and the average production is about 1,023 kg/ha. During 2005-06, the area under shrimp production is 8,172 ha and the production is about 9,739 with an average of 1,192 kg/ha.

In Orissa, the production of shrimp and the total area under shrimp production are positively related. The standard deviation has been very high meaning the production has not been consistent over the years. The reasons may be plenty. But there existed a positive relationship between the total production and the total area meaning that the shrimp farming has been managed profitably. But like many other states, there existed a negative relationship between the productivity and the total area under shrimp production. Solutions are to be found to solve the negative trend between the productivity and the area under production. About 30 per cent of the total area is under small farms of less than 2.0 ha (3,815 ha); 7.5 per cent of the area in farms of 2.0-5.0 ha size (941 ha); 57 per cent of the area in farms of above 5 ha size; and 5.5 per cent of the area is under corporate farms.

12.3.11 Shrimp Production in West Bengal

West Bengal has the largest potential area (4,05,000 ha) for Brackishwater farming. However, so far about 48,444 ha have only been developed for Brackishwater farming (Government of West Bengal, 2001). Excepting for 4,678 ha under improved farming, the remaining area falls in the category of traditional type with trap and culture, locally known as bheries.

Presently, the average production is about 840 kg/ha (MPEDA, 2005). During 1991-92, the area under shrimp farming was 33,910 ha and produced 15,800 tonnes with an average of 466 kg/ha. During 2005-06, the area under shrimp production is about 50,425 ha and yielded 42,336 tonnes with an average of about 840 kg/ha.

In West Bengal, a different situation was noticed. The total production and also the productivity had very strong positive relationship with the total area under shrimp production. In contrast with other states such as Karnataka, West Bengal shrimp farmers are managing the farms profitably. They excelled in maximizing the output and also per unit area of shrimp production and other states may have something to learn from the experience of West Bengal.

According to the recent estimates of the Government of West Bengal, 18,479 ha (38%) of the total area was in farm holdings of less than 2.0 ha; 4,237 ha (9%) under 2.0-5.0 ha and the remaining 25,728 ha (53%) in farms of above 5.0 ha. All the shrimp farms are creek-based and are within the CRZ zone. With the advance of scientific farming in the early seventies, the traditional farmers have also resorted to selective stocking with improvement in production levels.

12.4 DISTRIBUTION OF SHRIMP FARMS ACCORDING TO HOLDING SIZE

The number of farms under land holding categories of less than 2.0 ha, between 2.0 and 5.0 ha, between 5.0 and10 ha and more than 10 ha in the different maritime states, is presented below. The data shows that more than 90 per cent of the aqua farmers own less than 2.0 ha water area. It is seen from this data that farms below 5.0 ha occupy more than 60 per cent of the total area of shrimp farms in the country.

The compression of the regression coefficient reveals that the proportionate increase in the production per increasing unit area has been the maximum in Tamil Nadu being 1.5 tonnes per increase of 1 hectare in shrimp production followed by Karnataka. Whereas the

increase has been 0.79 tonnes in West Bengal, 0.77 tonnes in Orissa followed by Goa 0.73 tonnes, Andhra Pradesh 0.65 tonnes, Gujarat 0.32 tonnes. A negative trend has been noticed in Kerala and Puduchery. In Maharashtra the change in production per unit change in area has been significantly negative.

12.5 RECOMMENDATIONS AND CONCLUSION

Shrimp farming raises a host of problems because of its intricate linkages with the environment and socio-economic fabric of the people who practice and also related to it in one way or the other. The industry itself, together with allied institutions, can be expected to advance rapidly towards finding partial solutions to many of the technical, financial, marketing and administrative problems that it is confronting, as it has been doing during the past few decades. The present research suggests, however, that the industry is not likely to solve, or even devote substantial resources towards finding solutions to, many of the broader social and environmental problems associated with its continued growth. These involve power relations in the whole society, livelihood and health issues for local residents, concerns about the future health of the planet's natural environment, as well as the longer term economic sustainability of the industry. These issues have to be confronted jointly by public institutions and NGOs.

The many dimensions of the social and environmental impacts of the shrimp industry, as well as their institutional and policy implications, require an interdisciplinary approach involving collaboration among multiple stakeholders. The recommendations have been grouped into four categories i.e. Policy and Planning, Institutional, Environmental and Technical.

These recommendations would be useful for administrators, planners and political leaders, educators and non-governmental organizations for better management. The research could contribute to efforts to prevent the expansion of the industry in unsuitable social and environmental settings and to mitigate the adverse social and environmental impacts of existing and projected installations.

12.5.1 Policy and Planning

- As can be seen from the study, the main reason for current conflicts in coastal shrimp aquaculture development relates to improper site selection and poor design of the shrimp ponds. Hence, sustainable

shrimp farming is possible if only the planning process is scientifically carried out with due regard to ecological and social needs.

- Accordingly, proactive coastal zone planning with the careful selection of appropriate locations may assist in sustainable development of shrimp farming in India and avoid many of the problems experienced elsewhere.
- Since improper site selection as been identified as major reasons for unsustainable growth of shrimp farming during the course of the present study, a total review of shrimp farming at village, district and state level should be conducted by empowered technical committees for each State. Based on this review, a long-term plan of action for orderly and sustainable growth of shrimp farming should be drawn.
- In view of the above, the State level committees should give more consideration to the Coastal Zone Management Plans. In CRZ-I, no new shrimp farms should be allowed. In CRZ-III, the committee should take into account existing concentrations of shrimp farms and the existence of social and environmental conflicts.
- In view of the competing and conflicting demands of the stakeholders of the coastal areas so evident during the course of the study, management procedures need to be adopted in shrimp farming which would give careful consideration to the capacity of the environment to sustain eco friendly shrimp culture balanced against the needs of other users of coastal resources, which would certainly avoid many of the adverse environmental impacts that are associated with aquaculture.
- As can be seen from the study, unfortunately, not much data is available on the environmental impact of shrimp farming and little effort has been made to define criteria for sustainability. Hence R&D efforts should be targeted to evolve suitable methods for sustainable and eco-friendly coastal shrimp culture programmes.
- As the Aquaculture Authority has the maintenance of ecology as its prime guiding factor (Government of India, 1998), there needs to be equal consideration of environmental and fisheries interests in the permit system.
- The study exemplifies the need for a license for all farms, including existing farms which should be made mandatory to continue operations.
- The Aquaculture Authority would need to consider the closure of aquaculture farms that have been set up in severe violation of Indian law.

- The questionnaire survey has brought out the need for appropriate regulation and enforcement mechanisms for pond construction and management. Mangrove protection already evolved in some places in East Godavari District in Andhra Pradesh can be followed for all the mangrove areas where shrimp farming is under practice. Simultaneously, wise use of wetlands should be evolved and put in place.
- The corporate sector should come forward to share the benefits of shrimp farming with local people instead of alienating them.

12.5.2 Institutional

- The study revealed the absence of an Institutional mechanism to provide support to small and marginal farmers. An appropriate institutional and infrastructure support mechanism need to be provided to small and marginal farmers for access to resources such as land and water and also to markets.
- Lack of coordination among the relevant line departments at the State Government level has led to unsustainable shrimp farming as is revealed during the questionnaire survey with multiple stakeholders. Hence, State level committees of the Aquaculture Authority should be formed wherever not in existence and the existing committees are strengthened through increased involvement of the State departments of agriculture, environment and forests in addition to fisheries department. They are given sufficient means to carry out site investigations of all new applications and to conduct regular monitoring visits without prior notice to existing aquaculture farms. The committees should have the authority to close shrimp farms in case of severe violation of environmental or social regulations.
- During the course of the study, many stakeholders particularly the small and marginal farmers have suggested the setting up of a Shrimp Farmers Development Agency/Society especially in those districts where shrimp farming is prevalent. This may also be useful for provision of civic facilities such as drinking water facility etc through contribution of a small amount by the shrimp farmers towards development fund.
- Another innovative suggestion which has come up during the course of the study is to form coastal ‘aquaculture clubs’ whose main aim should be to disseminate the current information on diseases, environmental pollution, latest techniques, etc and ultimately to develop sustainable models of shrimp aquaculture.

- As many instances of blocking the approach of fishermen to the fish landing center and other places have come to the notice of the scholar during the study, it is recommended that a common approach to fish landing center/burial ground etc may be provided while designing the farm layout.
- Many of the small and marginal farmers, during the course of the study in general and questionnaire survey in particular, have expressed that the commercial banks are not coming forward to provide credit facilities to them for shrimp farm development. As the lack of credit facilities has been identified as one of the bottlenecks for the growth of shrimp farming, Development Banks such as NABARD should come forward to advance loans for needy small and marginal farmers to develop required infrastructural facilities and to facilitate growth of shrimp farming.
- As the study revealed that the small and marginal shrimp farmers in particular are being exploited by the middle men, market linkages need to be provided to them to enable them to access markets without depending upon the middlemen.

12.5.3 Technical

- Technical support need to be provided to the small and middle level entrepreneurs and the government may facilitate the development of shrimp farming industry and also ensure that adverse environmental and social impacts are suitably addressed.
- While making efforts to increase the productivity to the tune of 5 MT/ha/crop and above, it is suggested that the farms located in the seafront where quantity and quality of water is not a constraint, extensive/semi-intensive farms with production of not less than 4-5 MT/ha/crop may be planned.
- As it was noticed during the study that the shrimp culture wastewater is not treated before the same is discharged. Hence, there is an urgent need to put up an effluent treatment system before the pond water is discharged. It is recommended that not less than 7 per cent of the total cultivable water spread area may be used for effluent treatment system and enabling the discharged water to stay at least for a period of 24 hours before being let out into the main water source, enabling proper settlement of particles.
- Though aquaculture wastewater is considered as less harmful compared to the effluents of other industries, it should be treated through bio-ponds. Aquaculture waste is rich in nutrients and can be

utilized by integrating it with other aquaculture/agriculture production system.

- Proper peripheral drainage should be provided around the farms. The saline water intake and effluent discharge points should not be located in close proximity to agricultural fields and natural water bodies to guard against degradation of agricultural land, loss of fish catch, degradation of fragile costal land.
- Cultivation of species such as Clam, Mussels, milk fish, Tilapia, seaweeds may be done in the effluent water system which will generate additional income and will also hasten utilization of biodegraded wastes.
- In order to hasten the biological oxidation process, aerators may need to be introduced in the effluent ponds.
- Construction of cold storage-cum-ice factories may be encouraged.
- Laying of long distance pipelines for pumping sea water, construction of jetties into the sea, usage of ground water and fencing of farms should be prohibited.

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13

Molecular Response to Stress in European Sea Bass (*Dicentrarchus labrax*)

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13.1 INTRODUCTION

There is increasing public, governmental and commercial interest in the welfare of intensively farmed fish and stocking density has been highlighted as an area of particular concern (Ellis *et al*., 2002). Protecting the welfare of farmed animals is a central requirement of any animal-rearing system. Animal welfare involves the subjective feeling of animals and the experience of 'pleasure', 'pain', 'frustration', 'hunger' or other states and it is therefore difficult to define and to measure (Huntingford, 2002; Kristiansen and Juell, 2002).

Stress resulting from husbandry practices represents a major influence on both the health and welfare of farmed animals. The development of appropriate environmentally related husbandry practices can minimize the stress on farmed fish, reduce the incidence of diseases, and maintain high standards of animal welfare. Knowledge of these stress levels, therefore, represents a key parameter in achieving and maintaining standards of good fish husbandry and welfare.

In addition to several indicators that have been proposed for assessing fish welfare, molecular biomarkers directly indicating gene activity may have the characteristics for being useful early indicators. A biomarker is defined as any biological response (ranging from molecular through cellular and physiological responses to behavioural changes) to a

stress factor measured inside an organism indicating a deviation from the normal state that cannot be detected in the intact organism. In order to assess the exposure of fish to environmental stress conditions, it was suggested that a suite of biomarkers be examined, including evaluation of endocrine parameters.

The generalized stress response in fish has been broadly categorized into primary, secondary and tertiary response (Mazeaud *et al*., 1977). The 'primary' response starts with a blood increase of neuroendocrine/endocrine factors such as hormones, catecholamines and cortisol (Gamperl *et al*., 1994). The 'secondary' response comprises various hormone-induced biochemical and physiological effects, that result, for instance, in the alteration of haematological parameters such as glucose concentration (Vijayan *et al*., 1994, 1996, 1997). The 'tertiary' response is associated to the involvement of 'fish social life' that may result in appetite loss, compromised anabolic processes (reduced growth), reduced reproductive capability and frequent occurrence of infective pathologies (Iwama *et al*., 1998).

The significance of cortisol, which is frequently used to describe the condition of fish together with other direct parameters such as haematocrit, blood glucose or total haemoglobin, may be limited when the chronic stress of rearing fish at high densities is concerned (Van Weerd and Komen, 1998). The results of previous studies investigating whether increased stocking densities activates the stress response are conflicting, depending upon the species used and the experimental design (Tort *et al*., 1996; Procarione *et al*., 1999). These findings suggest that the use of plasma cortisol as an indicator of chronic stress may not be very informative, in particular, due to the acclimation of the interrenal gland during chronic stress and the influence of negative feedback mechanisms on the HPI axis (Rotllant *et al*., 2000). Thus, besides cortisol, other biomarkers can serve as indicators of stress levels in commercial farming situations (Gornati *et al*., 2004).

Our research in sea bass (*Dicentrarchus labrax*) focuses on identifying valid molecular markers capable of providing useful information about fish welfare. This species is of great interest for Mediterranean aquaculture as it is an excellent food fish with high commercial value. Accordingly, in our studies reviewed here we have isolated the complete cDNAs coding for some physiologically relevant stress-related proteins in this teleost, and then have assessed the impact of different stress conditions, such as high rearing density or acute and chronic hypoxia on their mRNA levels by real-time PCR.

13.2 GLUCOCORTICOID RECEPTOR

The stress response is a remarkably potent mechanism for resisting stress characterized by a number of endocrinal and metabolic changes. In teleosts, stress activates the hypothalamus-pituitary-interrenal (HPI) axis, leading to a rapid release of adrenocorticotropic hormone (ACTH) into the blood stream and a subsequent secretion of the glucocorticoid hormone cortisol by the interrenal tissue, the tissue analogous to the adrenal cortex in mammals. Fish do not possess a discrete adrenal gland as in mammals, and the steroidogenic cells -called interrenal cells- are distributed in the head-kidney region, mostly along the posteriorcardinal veins and their branches. These steroidogenic cells lie in close proximity to the chromaffin cells (which secrete catecholamines), raising the possibility of a paracrine control in the release of these hormones.

The effects of cortisol in fish consist in mediating stress-induced hyperglycemia, which is crucial for supporting the increased energy demand associated with stress, through activation of phosphoenolpyruvate carboxykinase (PEPCK) and gluconeogenesis (Hanson and Reshef, 1997; Mommsen *et al*., 1999; Saplosky *et al*., 2000). Cortisol released into the circulatory system enters cells by passive diffusion or is facilitated by a carrier-mediated process (Vijayan *et al*., 1997). Inside the cell it binds to a high-affinity cytosolic glucocorticoid receptor (GR), which acts as a ligand-dependent transcription factor to control and regulate gene expression (Evans, 1988). Receptor number or affinity may directly influence the degree of reactivity of target cells (Vanderbilt *et al*., 1987).

In teleosts, in addition to other processes traditionally thought to be mediated by the mineralocorticoids, such as salt balance, glucocorticoids, including cortisol and corticosterone, are known to mediate the stress response (Fuller *et al*., 2000). Teleosts lack the mineralocorticoid aldosterone which in mammals, regulate blood pressure principally by controlling sodium retention in the kidney. The effects of these two classes of corticosteroids are mediated by two related intracellular receptors, mineralocorticoid receptor (MR) and glucocorticoid receptor (GR). There is a substantial overlap in the pharmacology of these two types of receptors (Greenwood *et al.*, 2003) and cortisol binds to and induces the transcriptional activity of both. However, most cortisol activity is via GR. The presence of a MR has been recently reported by Colombe *et al*. (2000) in trout; it remains, however, to be seen if it is really involved in salt and water balance in teleosts. Ducouret *et al*. (1995) demonstrated in trout that the GR is the major or functional

cortisol receptor because the translated product of GR cDNA has ligand affinities very similar to those of cortisol receptors, as demonstrated by the classical binding assay (specific binding of radiolabelled cortisol to receptor preparations). In tilapia Tagawa *et al*. (1997) found a relatively higher expression of cortisol receptor mRNA in the gills and a medium level in the kidney. Furthermore, RU486, a specific blocker for GR but not for MR in mammals (Philibert, 1984), inhibited the osmoregulatory effect in Atlantic salmon (Veillette *et al*., 1995; Vijayan and Leatherland, 1992).

13.3 3-HYDROXY-3-METHYLGLUTARYL-COENZYME A REDUCTASE (HMGCOAR)

HMGCoAR is a particularly well studied enzyme that catalyses the reduction of HMG-CoA to CoA and mevalonate which is the rate-limiting reaction in the cholesterol *de novo* synthesis. HMGCoAR is the target of statins, a class of drugs highly efficacious in controlling hypercholesterolemia; these inhibit cholesterol biosynthesis by blocking the access of the substrate to the active site of the enzyme (Istvan, 2002).

Cholesterol is the precursor of cortisol, the hormone secreted by interrenal tissue (equivalent to the adrenal glands for mammals) in response to a variety of stress conditions, that is a widely used biomarker in aquaculture. In fact, circulating levels of cortisol are elevated in fish during periods of stress in order to maintain the homeostasis (Momsen *et al*., 1999).

HMGCoAR gene expression has been shown to be influenced by several factors. The levels of HMGCoAR mRNA are reduced by procaine (Xu *et al*., 2003), fenofibrate, a potent hypolipidemic agent (Guo *et al*., 2001), oxysterols (Kisseleva *et al*., 1999), monoterpenes (Peffley and Gayen, 2003), cholesterol-enriched diet and starvation (Sato *et al*., 2003); conversely, they are increased by pathological conditions, such as tumors (Hentosh *et al*., 2001) and renal failure (Chmielewski *et al*., 2003), by Cu++ exposure (Svensson *et al*., 2003), growth hormone (Machado *et al*., 2003) and red wine (Pal *et al*., 2003). Moreover, the expression of HSP and HMGCoAR resulted increased after heat shock in the study of Zager and Johnson (2001).

13.4 HYPOXIA INDUCIBLE FACTOR

Oxygen is essential to life for most organisms, but changes in the environment can reduce the availability of oxygen. In general, hypoxia even for brief periods can be detrimental or fatal to humans and most mammals as they possess only little tolerance to anoxia and their tissues are normally debilitated by any prolonged lack of O_2 (van der Meer *et al*., 2005). However, certain vertebrate species have evolved to subsist on low amounts of oxygen for prolonged periods owing to a range of specially developed physiological mechanisms known as the 'hypoxia response' (Nikinmaa, 2002; Nikinmaa and Rees, 2005). Fish species that live and survive in environments with low and/or variable oxygen levels represent an example of this. Thus, it can be expected that these animals have highly evolved mechanisms for surviving aquatic hypoxia that are perhaps more sophisticated than those of the more extensively studied terrestrial mammals.

Acute decreases in water oxygen concentrations may occur in intensive fish farming as well, especially when fishes are reared at high densities. In this respect, considerable attention has been paid to oxygen, as low ambient O_2 concentrations are known to affect growth, food intake and the physical state of fishes (Jobling, 1994).

Several studies have investigated the adaptive response of fish to low oxygen levels from a morphological/physiological point of view. For example, it has been shown that, in the cyprinid, crucian carp (*Carassius carassius*), which is especially suited to withstand periods of anoxia, hypoxia leads to dramatic and irreversible, gross morphological changes (Sollid *et al*., 2003). In sea bass exposed to hypoxia condition, gills were disorganized, the vascular lumen was reduced and the lamellae showed apical blebs and outer epithelial detachment (Rinaldi *et al*., 2005). In cichlid species, chronic hypoxia caused elongation of gill filaments and increased the size of secondary lamellae (Chapman *et al*., 2000). Another study showed that zebrafish embryos can survive even in the total absence of oxygen (anoxia, 0% O_2) (Padilla and Roth, 2001). In that study, zebrafish entered a state of suspended animation in which cell division stopped and developmental progression ceased after 24 hrs of 0% oxygen. However, the molecular responses to hypoxia have not been studied extensively in fish, even though these animals are ideal models for these studies.

In fish a complex set of physiological and biochemical alterations are employed to cope with hypoxia stress. These strategies include decreased metabolic rate (DallaVia *et al*., 1994), increased ventilation rate,

hematocrit and haemoglobin O_2 affinity (Jensen *et al*., 1993), and increased anaerobic respiration (Virani and Rees, 2000). Many of these adjustments depend to a large extent on changes in the expression of genes that encode diverse groups of physiologically relevant proteins. Gracey *et al*. (2001) recently identified alterations in the expression of over 120 genes in hypoxic fish (*Gillichthys mirabilis*), whereas in mammals, hypoxia-induced changes in the expression of a wide range of genes were reported (Bruick, 2003), including numerous genes homologues to those observed in fish. Examples include genes involved in glycolysis, gluconeogenesis, iron metabolism, cell survival and proliferation, translational machinery, and muscle contraction (Semenza, 1999; Wenger, 2002; Gracey *et al*., 2001).

In mammals, where HIF has been extensively studied, genes that are induced by hypoxia appear to share a common mode of transcriptional regulation. This induction depends upon activation of a transcription factor, the hypoxia inducible factor-1 (HIF-1). Under hypoxic conditions, the HIF-1 binds specifically to a consensus sequence (5'- RCGTG - 3') known as the hypoxia-responsive element (HRE) in the promoter or enhancer of various hypoxia-inducible genes (Semenza, 1999; Wenger, 2002). HIF-1 is a heterodimer composed of α and β subunits. HIF-1 α is generally found to be constitutively expressed in the nucleus and to be insensitive to changes in O_2 availability, whereas stabilization of HIF-1α and its nuclear accumulation are acutely regulated by hypoxia (Uchida *et al*., 2004).

The most intriguing feature of HIF-1α is the presence of a so-called oxygen-dependent degradation domain (ODD) that can confer hypoxic stabilization to HIF-1α (Huang *et al*., 1998; Pugh *et al*., 1997; Srinivas *et al*., 1999). Under normoxic conditions, this domain receives a signal (hydroxylation of two conserved proline residues Pro-402 and Pro-564) that increases the affinity of HIF-1α for the Von Hippel Lindau tumor suppressor protein, which is part of an E3 ubiquitin-ligase complex that targets the protein for proteasomal degradation. Degradation of the α subunit disrupts HIF-1, thus inactivating the protective response. When oxygen levels fall, however, HIF-1α degradation stops; it translocates to the nucleus and dimerizes with HIF-1β thus allowing the active transcription factor HIF-1 to accumulate and go into action. Although the term 'hypoxia–inducible factor' implies increased production at low oxygen tension, in this way hypoxia, in fact, slows down the destruction of HIF-1α.

Since the initial characterization of HIF-1α in humans (Semenza and Wang, 1992), several additional cDNAs have been isolated in different

vertebrates, whereas orthologues from fish have only been identified in a few species. The sequence of the first fish HIF-1α was characterized in rainbow trout cells (Soitamo *et al*., 2001), and its DNA sequence showed a 61% similarity to that of human HIF-1α. In subsequent years, a number of complete or partial HIF-1α sequences were obtained from scale-less carp (*Gymnocypris przewalskii*) (Cao *et al*., 2005), grass carp (*Ctenopharyngodon idella*) (Law *et al*., 2006), zebrafish (*Danio rerio*) (Rojas *et al*., 2007), Atlantic croaker (*Micropogonias undulatus*) (Rahman and Thomas, 2007) and European perch (*Perca fluviatilis*) (Rytkönen *et al*., 2007).

Understanding how hypoxia alters gene expression in fish (Ton *et al*., 2003) will likely contribute to our knowledge of how vertebrates respond to hypoxia in general and illustrate the dynamic interactions between genes and environment. Thomas *et al*. (2007) have made an important contribution to our understanding of the molecular basis of the adaptive response to low oxygen levels in hypoxia tolerant species, whereas studies on hypoxia-sensitive species are still lacking.

13.5 MOLECULAR CHARACTERIZATION OF TARGET GENES

At the beginning of our research, the coding sequences of glucocorticoid receptor (GR), 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCoAR), and hypoxia inducible factor (HIF-1α and were not available in public databases for *D. labrax*; in fact, despite the high commercial interest in this animal, information on its genome, transcriptome, or proteome is minimal. Considering this lack of information, we aimed first to obtain the open reading frames of each gene. A BlastN search (http://www.ncbi.nlm.nih.gov/BLAST/) was performed on the complete, nonredundant Genbank nucleotide database for orthologues of GR, HMGCoAR, and HIF-1α in other fish species. A multiple sequence nucleotide alignment was then carried out on the coding sequences found for each gene, and a strategy based on regions of strong nucleotide conservation was used to design the primers. In the case of GR for example, primer design was based on the multiple alignment of three teleost GR coding sequences available on the NCBI Genbank database: *Paralichthys olivaceus* (accession no. AB013444), *Astatotilapia burtoni* (accession no. AF263740) (Greenwood *et al*., 2003) and *Onchorhynchus mykiss* (accession no. Z54210 and AY495372). These presented several conserved regions within the sequence where primers

could be reasonably designed (Terova *et al.*, 2005). The location and orientation of the primers designed for the sea bass GR and HIF-1α amplification are presented in Figure 13.1 and 13.2 respectively, whereas their sequences are presented in Table 13.1.

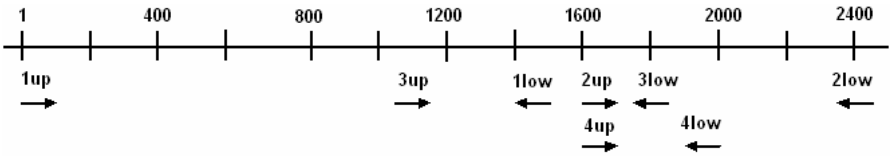

Figure 13.1: Schematic diagram of the PCR amplification of sea bass glucocorticoid receptor. The scale denotes numbers of nucleotides of the cDNA sequence. Location and orientation of the primers are indicated by arrows.

```
                HIF FW1
atggacacaggagttgtcccagaaacagaaagcagggtgagctcggagcggaggaaggagaagtcgagggat   72
 M  D  T  G  V  V  P  E  T  E  S  R  V  S  S  E  R  R  K  E  K  S  R  D

          GSP3 RACE 5'
agactcaccatcagctacctgcgcatgaggaaactgctcaacactgatgagccaatgaaagacgaggaaaca  252
 R  L  T  I  S  Y  L  R  M  R  K  L  L  N  T  D  E  P  M  K  D  E  E  T

                                GSP2 RACE 5'
gacctggatctccagttaaacagctcctacctaaaggctctggagggttttctcatggtgctgtctgaagac  324
 D  L  D  L  Q  L  N  S  S  Y  L  K  A  L  E  G  F  L  M  V  L  S  E  D

 GSP1 RACE 5'                                                HIF Fw2
atacatccctgtgaccaagatgagctgagggagatgctggtccacagaacaggctctaaaaagtccaaggaa  483
 I  H  P  C  D  Q  D  E  L  R  E  M  L  V  H  R  T  G  S  K  K  S  K  E

                          HIF RV1
gtctgcacaggccagtaccggatgttggccaagagaggaggctttgtgtgggtggagacccaagccactgtc  972
 V  C  T  G  Q  Y  R  M  L  A  K  R  G  G  F  V  W  V  E  T  Q  A  T  V

                                HIF Fw3
accatcatctccctggacttcagctgccctgattcagagatcccgatgatgaacgatgttcctgtctacaat 1392
 T  I  I  S  L  D  F  S  C  P  D  S  E  I  P  M  M  N  D  V  P  V  Y  N

                          HIF RV2
gatatgagctcagatttcaaactcgacttggtggagaagctgtttgccattgatacagagcccaagaccccc 1656
 D  M  S  S  D  F  K  L  D  L  V  E  K  L  F  A  I  D  T  E  P  K  T  P

                                HIF RV3
ccacagctcacccgtgacgactgcgaggtcaacgccccctacagggtcgtcagtacctgctgcaggggggag 2232
 P  Q  L  T  R  D  D  C  E  V  N  A  P  L  Q  G  R  Q  Y  L  L  Q  G  E

gagctgctgcgcgctctggaccacgtcaactgagtcattgcttagcctgctcactgctgaactagactggac 2304
 E  L  L  R  A  L  D  H  V  N  -
actacaatttctccctcatattgtcactccctctcagctcactgctgaactagactggactctgtttgagtt 2376
tgactatagttttgctgctgtgtgggcacatcatgtgatatctgcagcattccaccgtgacaaacaccatgg 2460
cagcatttgggttttggtgcaagggattcagacatcatgagatgtccaattgttgttttttttcgttttgtt 2532
tcctctctcttcggggagtctctcagcacacagcgcctcgacagatgacccttttccagcaggtcaccacag 2604
aacctggcgctactgccagcagtgtattgtaagcttcagtcgcacaatttatattttcttaaaaagaaaaat 2676
attaccagcaatatatattaagccttttttaaagtcgttttaatgggatttgaacaatttttatttttcctccc 2748
atacttgtatgttgtagtactcgtacctaacaagatgtctttagtcgagaagtatgaacatattgtttaaac 2820
cttaggtggtttgaatgttatctccatttaatagttatcagtctttgaggtaaatgcatttaattgtgtagc 2892
agtccagtacatgtcactttattactttactgtaagtgtgtgttactgtacatataccagggaaatttgaat 2964
ttactcagtatggcttcacagttttgactctcaaaatgaaatatgagtcttcactatttttgtttgcttctc 3036
atcatggagaattggcatttttctcttaacatgaagtggattcatgtttttggttttttaatgtgcgtgacat 3108
actcaaaatgagtctgttttaaagtaagtaggatctcgacaattcatatacttgtgtgatcatagtagcatt 3180
cgtgagttgtcaaacttgtttgcttagttaatggtagtcagttcaataccattgatccctgccatattgttg 3252
ggtctctcatgcttcttgttgctgtctaacattaaatggctttaatgaaaaaaaaaaaaaaaaaa        3300
```

Figure 13.2: The nucleotide sequence of seabass (Dicentrarchus labrax) HIF-1α (accession no. DQ171936), with the deduced amino acids shown below the sequence in single-letter code. Nucleotides are numbered to the left. The locations of the primers used in PCR of the full-length transcript and in the 5′ or 3′ RACE are also indicated by solid and broken horizontal arrows, respectively.

Table 13.1: Sequences and Tm of primers used for glucocorticoid receptor sequencing

Primers	Sequence 5′ – 3′	Tm (°C)
GR 1up	ATG GAT CAG GGT GGA CTG AA	57.3
GR 1low	TCTGAACACACCAAACAGATCTTATG	60.1
GR 2up	GGA CCT GGC TAC CAC TAC AGA	61.8
GR 2low	GAT GAC TGG AAC AGG CAT GTT	57.9
GR 3up	TTC TGT CAG CTT CTC CAG CTC	59.8
GR 3low	TCACAAAGGTGTAGAAACAGATCTG	59.7
GR 4up	CCT GAT CTT GTC ATC AAC AAA GAA	57.6
GR 4low	TCA TTT CTG ATG AAA CAG CAG A	54.7
Actin left	GAA GAT GAA ATC GCC GCA CT	57.3
Actin right	TTA GAA GCA TTT GCG GTG CA	55.3

Total RNA was extracted from different sea bass tissues. The quantity of the RNA was calculated using spectrophotometry. The integrity and relative quantity of RNA was checked by electrophoresis. After extraction, an aliquot of total RNA was reverse transcribed into cDNA. PCR amplifications were performed amplifying an aliquot of the resulting cDNA with GoTaq Polymerase. The annealing temperatures depended on the melting temperatures of the primer set used. An aliquot of each sample was then electrophoresed and bands were detected by ethidium bromide staining. The PCR products from GR, HMGCoAR, and HIF-1α primer amplifications were then cloned and subsequently sequenced.

Several cDNA fragments were obtained following this cloning strategy. Then, by connecting the sequences of the partially overlapping clones, partial coding sequences (~ 250 bp) of each gene were determined. The full-length cDNAs of each gene were subsequently isolated by 5'- and 3'- RACE and deposited in GenBank under the accession no. AY549305 for GR, AY424801 for HMGCoAR, and DQ171936, for HIF-1α.

13.5.1 Glucocorticoid Receptor

The isolated complete coding sequence of sea bass GR (2454 bp) encodes a 818 amino acid protein with a calculated molecular mass of 90 kDa.

The sequence (accession no. AY549305) (Terova *et al*., 2005) exhibited a high degree of identity with other GR sequences known in fish species (70-80% identity). A comparison of the amino acid sequence of sea bass and other GR proteins in fish species, showing the regions containing the highly conserved residues and the nine additional residues (Ducouret *et al*., 1995) specific for GR in fish species, is presented in Figure 13.3.

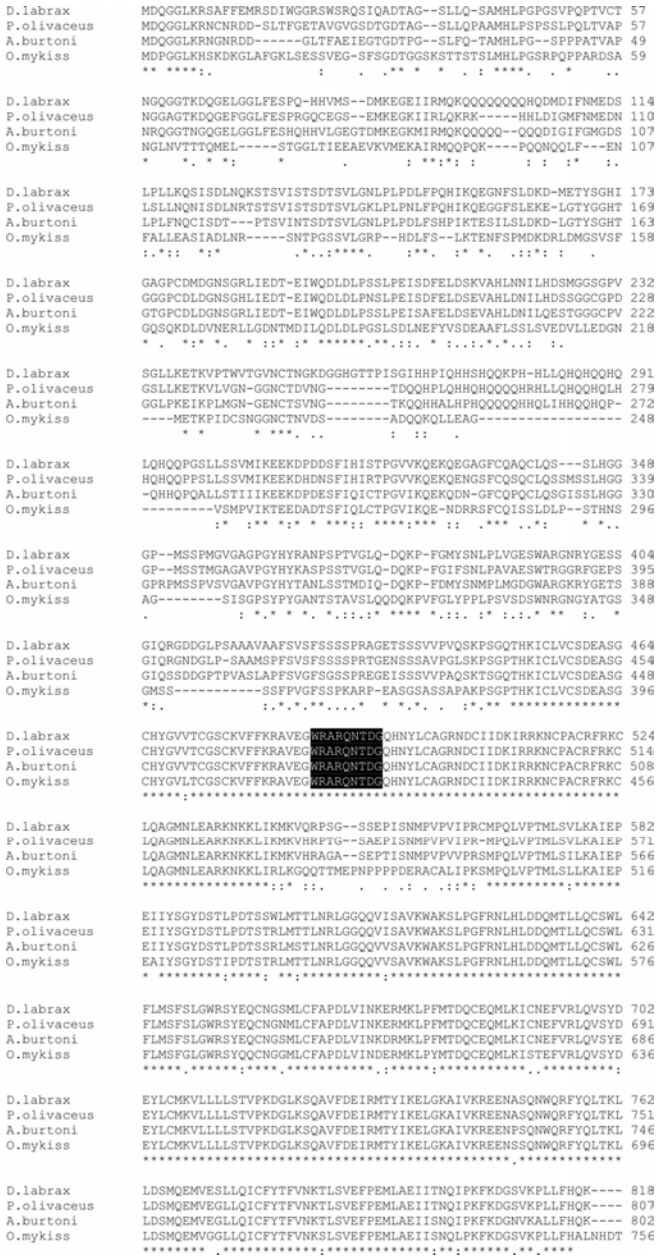

Figure 13.3: Alignment of the predicted amino acid sequence of sea bass (D. labrax) glucocorticoid receptor with other GR sequences of fish species Paralichthys olivaceus, Astatotilapia burtoni and Onchorhynchus mykiss. Gaps introduced for optimal alignment are indicated by dash. (*) indicates identical or conserved residues in all sequences, (:) conserved substitutions and (.) semi-conserved substitutions. Black boxes with white letters represent the nine additional residues, specific for GR in fish species.

Dicentrarchus labrax GR has the 9 amino acid insertion between the two zinc fingers within its DNA binding domain, which is different from that of mammals. This insertion was first identified in the rainbow trout by Ducouret *et al*. (1995) and subsequently has been described in *Oreochromis mossambicus* (Tagawa *et al*., 1997). Another teleost, *Paralichthys olivaceus*, which belongs to a different order, possesses the same peptide insertion WRARQNTDG in its DNA binding domain. Thus, this additional exon, in comparison with mammalian GRs, may be present in all teleosts and, as it promotes greater DNA affinity in the GR, could have been selected to serve the large spectrum of cortisol functions in fish (Lethimonier *et al*., 2002).

13.5.2 HMGCoAR

The sea bass HMGCoAR (accession no. AY424801) (Gornati *et al*., 2005) exhibited a high degree of identity (76 to 77%) with other HMGCoAR sequences known in vertebrate species, such as *Oryctolagus cuniculus*, *Homo sapiens*, *Cricetulus griseus*, *Mesocricetus auratus*, *Rattus norvegicus*, *Mus musculus*, *Gallus gallus*, and *Xenopus laevis,* or invertebrates (echinoderms) such as sea urchin *Strongylocentrotus purpuratus* (Table 13.2).

Table 13.2: Identity percentage of multiple sequences alignements of HMGCoAR of Oryctolagus cuniculus (O.c.), Homo sapiens (H.s.), Cricetulus griseus (C.g.), Mesocricetus auratus (M.a.), Rattus norvegicus (R.n.), Mus musculus (M.m.), Gallus gallus (G.g.), Xenopus laevis (X.l.), Dicentrarchus labrax (D.l.), and Strongylocentrotus purpuratus (S.p.)

H.s.	**95**								
C.g.	93	93							
M.a.	93	93	98						
R.n.	92	92	96	95					
M.m.	92	93	96	96	97				
G.g.	86	86	85	84	85	85			
X.l.	80	81	80	80	79	80	81		
D.l.	76	76	76	76	76	76	77	76	
S.p.	57	58	58	58	58	55	58	59	58
	O.c.	H.s.	C.g.	M.a.	R.n.	M.m.	G.g.	X.l.	D.l.

The N- and C-terminus portions of the sequence are the most conserved and are linked by an hydrophilic region that appears to be quite variable. The human HMGCoAR is known to possess an amino terminal portion of 339 aminoacids that resides in the endoplasmic

reticulum membrane and a 229 C-terminal portion with catalytic activity that extends into the cytosol where it combines with three other catalytic portions to form a tetramer (Istvan, 2002). The % identities increase from 82 to 86%, if the N and C terminal regions of *D. labrax* HMGCoAR are compared to the corresponding portions of the same enzyme of other vertebrates.

13.5.3 HIF-1α

The full-length cDNA for HIF-1α (accession no. DQ171936) (Terova *et al*., 2008) consists of 3317 base pairs (bp) carrying a single open-reading frame that encompasses 2265 bp of the coding region and 1052 bp of the 3' UTR, including a common (AATAAA) polyadenylation signal upstream of the poly(A) tail. Conceptual translation of the cDNA predicts a protein of 755 amino acids (aa) (Figure 13.2).

The predicted sea bass HIF-1α amino acid sequence shows extensive sequence similarity to human HIF-1α in the bHLH (basic helix-loop-helix domain), PAS A/B (Per-ARNT-Sim A/B domain), PAC (C-terminal to PAS motifs), N-TAD (N-terminal transactivation domain), and C-TAD (C-terminal transactivation domain), whereas there are few variations in the ODD (oxygen-dependent degradation domain) (Figure 13.4). The bHLH region, which is known to be responsible for DNA binding and dimerization, is critical as changes in gene expression under hypoxic conditions can only occur after HIF is bound to DNA. Amino acid substitutions in this domain are known to affect DNA binding to HIF-1 (Lando *et al*., 2000). In sea bass, a hypoxia-sensitive species, the amino acid that aligns with human position 28 in the bHLH domain is cysteine, like in salmonids, and not serine, like in the hypoxia-tolerant cyprinids. Rytkönen *et al.* (2007) were the first to hypothesize that the substitution cysteine/serine in this position correlates with a species-specific oxygen demand. It also coincides in our species, although the authors then concluded that such a correlation was not present in all the fish species they analyzed from a phylogenetic point of view. Several functionally important sequence motifs described in all known members of the HIF-1α protein family are also found in the sea bass HIF-1α. They include:

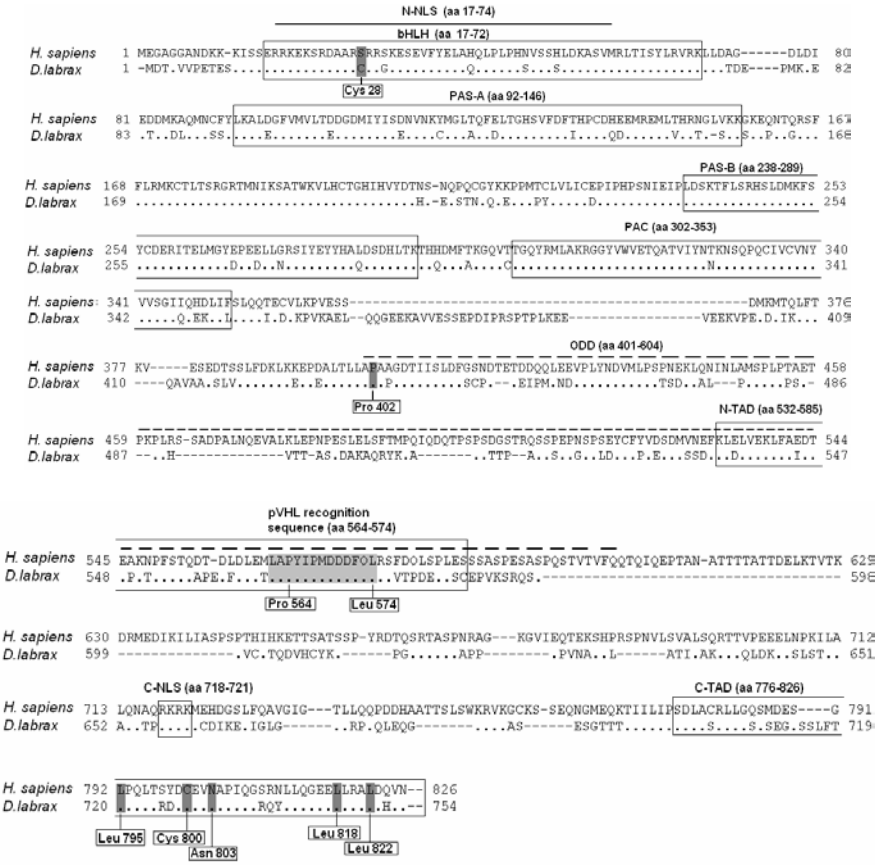

Figure 13.4: Alignment of the deduced aminoacid sequence of seabass (Dicentrarchus labrax) HIF-1α (accession no. DQ171936) with the HIF-1α related protein of Homo sapiens (accession no. NP-001521). Amino acids are designated by single-letter codes and are numbered to both sides. Dots indicate residues that are identical to human HIF-1α. Dashes indicate gaps introduced to facilitate alignment. The N-terminal nuclear localization signal (N-NLS) residues 17-74 (numbering according to human HIF-1α) and the oxygen-dependent degradation domain (ODD) domain are over lined. The basic helix-loop-helix (bHLH); Per-ARNT-SIM (PAS)-A and B; transactivation domain (TAD) N- and C-terminal; C-terminal nuclear localization signal (C-NLS) are indicated inside a box. The sea bass aminoacids that aligns with human position 28 (Ser), 795 (Leu), 800 (Cys), 803 (Asn), 808 (Gly), 810 (Arg), and 823 (Asp) are shadowed.

- The N-terminal nuclear localization signal (N-NLS) residues 17-74 (numbering according to human HIF-1α) that mediate nucleocytoplasmic trafficking of the HIF-1α protein (Ema *et al*., 1997; Luo and Shibuya, 2001)

- The highly conserved (Leu562-Ala-Pro-Tyr-Ile-Pro-Met-Asp569) sequence, which enables specific binding to von Hippel-Lindau (VHL) protein, and the subsequent ubiquitin/proteasome degradation of HIF-1α under normoxic conditions (Ivan *et al*., 2001; Pereira *et al*., 2003)
- Pro 402, a critical hydroxylation site that mediates HIF-1α degradation under normoxia (Masson *et al*., 2001)
- Leu-574, a molecular determinant of Pro-564 hydroxylation that modulates oxygen-dependent proteolysis of HIF-1α (Kageyama *et al*., 2004).

The overall high degree of HIF-1α sequence conservation through evolution is not only consistent with its essential role in various responses to hypoxia, but also suggests that its biological action may be equally well conserved. In fact, the mechanism of degradation and stabilization of HIF-1α protein is most likely the same in fish as in mammals (Soitamo *et al*., 2001), although in fish (rainbow trout and chinook salmon) stabilization occurred at much higher oxygen levels than in mammals, suggesting a role for oxygen-regulated gene expression in the normal physiology of fish.

13.6 PHYLOGENETIC ANALYSIS

To analyze the evolutionary relationship of sea bass GR, and HIF-1α with respect to other publicly available, related genes in other teleosts and in amphibian, avian, and mammalian species, we reconstructed phylogenetic trees. These analysis were computed by TREEFINDER, version from October 2005 (Jobb *et al*., 2004). This program computes phylogenetic trees from nucleotide sequences, using the widely accepted Maximum Likelihood method and a novel tree search algorithm. It also accepts incomplete site patterns in a data matrix and can reconstruct trees from partially overlapping sequences. In our case, 1000 bootstrap replicates were performed (expressed in % on the branch nodes) for each analyzed gene.

The results showed that sea bass glucocorticoid receptor is clearly in the branch of other teleosts such as *Paralichthys olivaceus* and *Astatotilapia burtoni* (Figure 13.5) (Terova *et al*., 2005). The clustering pattern of sea bass HIF-1α provided evidence that it is grouped with high bootstrap support in the lineage of other teleosts, sharing the highest homology with Atlantic croaker (*Micropogonias undulatus*) HIF-1α, whereas the amphibians (frog), birds (chicken), and the mammals

(rabbit, mouse, rat, mole rat, cow, pig, and human,) are grouped into three other distinct lineages (Figure 13.6) (Terova *et al*., 2008). Fish represent an extremely large and divergent vertebrate group. Among fish HIF-1α, great variations exist in homology. This is different from the situation observed in other vertebrate groups, such as mammals, in which homology among mammalian HIF-1α is very high. Sea bass HIF-1α is, in fact, most homologous to that of Atlantic croaker (*Micropogonias undulatus*), showing an approximately 87% aa identity. Homology with the rainbow trout (*Onchorhynchus mykiss*) and zebrafish (*Danio rerio*) is lower, being 71 and 61%, respectively, while homology with crucian carp (*Carassius carassius*) HIF-1α is even lower, with merely a 59% aa identity. Despite the great variation, fish HIF-1αs do form a distinctive group as compared to the HIF-1αs of other vertebrates, as revealed by the phylogenetic analysis shown in Figure 13.6. Here, the HIF-1αs of similar phylogenetic origin are clustered together. The HIF-1α of sea bass (*D. labrax*) and Atlantic croaker are most homologous to each other while the flounder, grass carp, and rainbow trout HIF-1α appear to form a distinctive subgroup among fish HIF-1α.

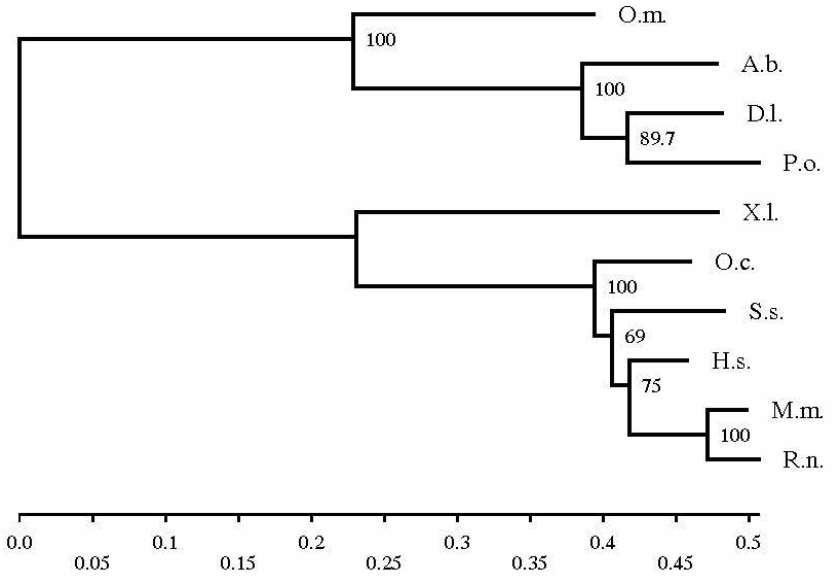

Figure 13.5 Phylogenetic tree comparing the nucleotide sequence of GR in Dicentrarchus labrax (D.l) with those of other species, i.e., Paralichthys olivaceus (P.o.), Astatotilapia burtoni (A.b.), Onchorhynchus mykiss (O.m.), Xenopus laevis (X.l.), Mus musculus (M.m.), Rattus norvegicus (R.n.), Homo sapiens (H.s.), Oryctolagus cuniculus (O.c.), and Sus scrofa (S.s.).

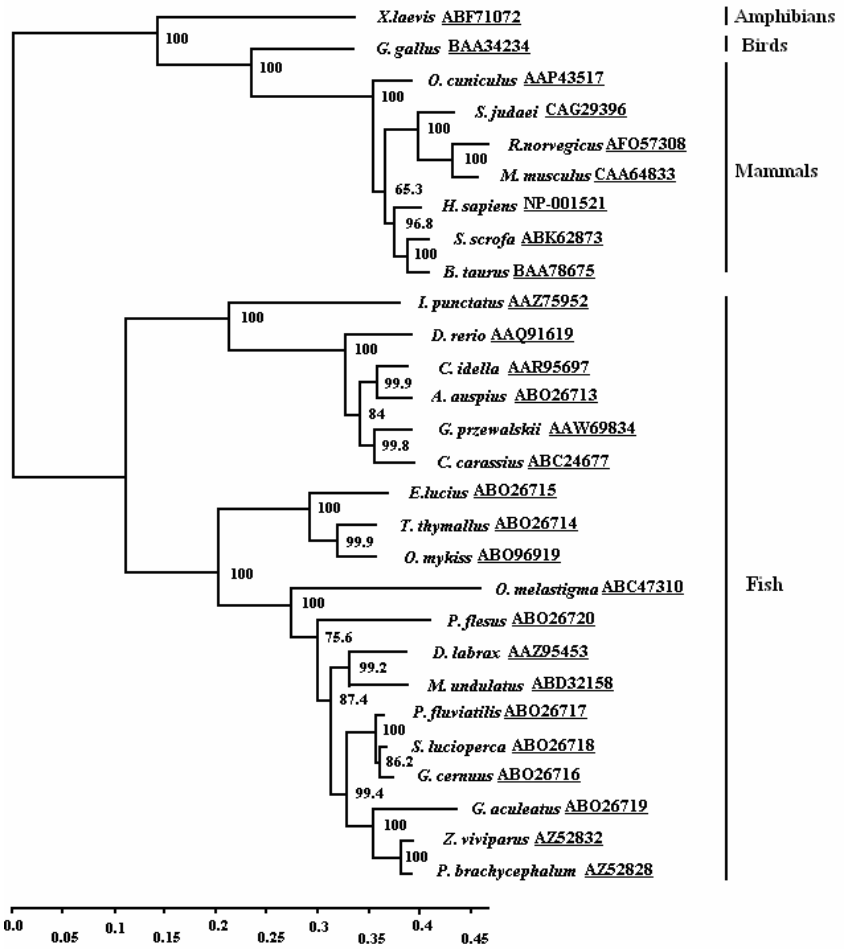

Figure 13.6: Phylogenetic tree comparing the sequence of HIF-1α in sea bass (Dicentrarchus labrax) with those of other vertebrate species. The scale bar refers to evolutionary distances in substitutions per site. The numbers at tree nodes refer to percentage bootstrap values after 1000 replicates. The numbers on the right hand side of the species name are GenBank accession numbers for HIF-1α in that species. The following species are considered for this analysis: asp (Aspius aspius); crucian carp (Carassius carassius); zebrafish (Danio rerio); channel catfish (Ictalurus punctatus); scale-less carp (Gymnocypris przewalskii); grass carp (Ctenopharyngodon idellus); rainbow trout (Onchorhynchus mykiss); Indian medaka (Oryzias melastigma); European flounder (Platichthys flesus); pike (Esox lucius); grayling (Thymallus thymallus); three-spined stickleback (Gasterosteus aculeatus); ruff (Gymnocephalus cernuus); perch (Perca fluviatilis); pikeperch (Stizostedion lucioperca); Atlantic croaker (Micropogonias undulatus); Antarctic fish (Pachycara brachycephalum); Antartic fish (Zoarces viviparous); African clawed frog (Xenopus laevis); chicken (Gallus gallus); mole rat (Spalax judaei); rabbit (Oryctolagus cuniculus); house rat (Mus musculus); rat (Rattus norvegicus); pig (Sus scrofa); cow (Bos taurus); and human (Homo sapiens).

The phylogenetic tree comparing the HMGCoAR aminoacid sequence of sea bass with those of other teleosts or vertebrate species was in agreement with the accepted phylogenetic relationships of the used species (Figure 13.7) (Gornati *et al*., 2005). It was computed by PHYLIP (Phylogeny Inference Package), Version 3.5, Copyright 1986-1993 by Joseph Felsenstein and the University of Washington, Seattle, WA. Sequences were bootstrapped 100 times, distance matrices were built by PROTDIST (Dayhoff matrix) and clustering was done by the UPGMA procedure of NEIGHBOR.

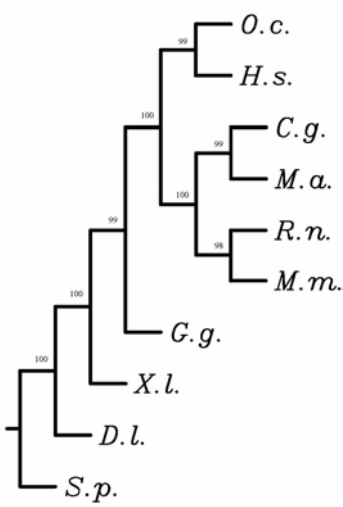

Figure 13.7: UPGMA tree of HMGCoAR from ten species, based upon the sequence comparison of 909 aminoacids (from the N-term of the protein). The original data set was bootstrapped 100 times, and the bootstrap values are reported for each node. Oryctolagus cuniculus (O.c.), Homo sapiens (H.s.), Cricetulus griseus (C.g.), Mesocricetus auratus (M.a.), Rattus norvegicus (R.n.), Mus musculus (M.m.), Gallus gallus (G.g.), Xenopus laevis (X.l.), Dicentrarchus labrax (D.l.), and Strongylocentrotus purpuratus (S.p.).

13.7 STRESS MANIPULATION OF GENE EXPRESSION

After the isolation of sequences of GR, HMGCoAR, and HIF-1α we assessed by real-time RT-PCR, the impact of high rearing density stress conditions and hypoxia, on their expression levels. For this purpose two different experiments were carried on.

13.7.1 High Rearing Density Stress

Fingerling sea bass obtained from Nuova Azzurro commercial hatchery (Civitavecchia, RM Italy) were reared, with inconsistent mortality, at a low biomass density (<10 kg/m^3) in a 4 m^3 fiberglass tank connected to a water recycling system supplied with about 24 water refillings per day. The salinity (obtained by adding salt Oceanfish 600LT from Prodac Int® to dechlorinated tap water) was 10 g/l. Sea bass is a strongly euryhaline marine fish that may be found under natural conditions at salinities ranging from freshwater to 90 g/L (Barnabé, 1990). Recent studies show good growth performances at salinities ranging from 10 to 37 g/L. A low salinity preference was shown for juveniles of these species (Saillant *et al*., 2003), while Dalla Via *et al*., (1998) reported good metabolic performances at salinities as low as 2 g/L.

Other water parameters were strictly controlled: temperature 20±1°C, PO_2 95–105% of the saturation value (obtained with pure O_2 insufflation), free CO_2 < 15 mg/l, N-NH_4^+ < 0.03 mg/l, total gas pressure 98–100% of the saturation value, N-NO_3^- < 20 mg/l, pH 7.2, and alkalinity 140–200 mg/l $CaCO_3$.

At an average size of 50 g, three populations were randomly selected and transferred to 200-l fiberglass tanks at different nominal densities, corresponding to 10, 80, and 100 kg/m^3, with the same environmental conditions and water refilling rate. Food was automatically distributed daily to ensure a 1.5% body mass ratio, with extruded pellet for marine fish (TROUVIT Power HQII®). Temperature, PO_2 and pH were continuously monitored, while other parameters were assessed weekly. Cortisolemia was checked on a monthly basis in blood samples with the aim of monitoring the stress status of the animals. For this, fish were anaesthetized with 3-aminobenzoic acid ethyl (MS222, 100 ppm) and the blood was drawn from the caudal vein with a heparinized syringe, in less than 1 min (usually 30-40 sec.). Catching an individual fish did not cause stress to remaining ones, as the fish from each experimental group were taken out of tank and punctured at the same moment by different operators. The blood was centrifuged (10,000 g for 5 min) and plasma was stored at –70°C for later hormonal analyses. Plasma cortisol was measured using an ELISA kit from IBL-Hamburg on a 96-well microplate reader (λ=450 nm). To maintain the nominal densities, calibrated sampling was performed weekly in each of the experimental tanks. After 3 months, during which no mortality was observed, three animals from each group were randomly sampled, and immediately sacrificed. Liver and brain were removed *in toto*, frozen in liquid N_2, and stored at -80°C

until molecular biology analysis. Samples of sea bass farmed at 50 kg/m^3, a density considered relatively high for commercial farming, were obtained from Agroittica Toscana (Piombino, Italy), a land-based farm that utilizes marine water and liquid O_2.

13.7.2 Hypoxia Stress

In this case sea bass of mixed sexes were obtained from a local fish farm in December 2004 and kept at our department, in Varese, Italy. Fish were stocked into long indoor tanks of 2500 L each and allowed to acclimate for 45 days before starting the trial. The tanks were connected to a sea water recirculation system, with strictly controlled water conditions: temperature 21.8±0.9°C, pH 7, total ammonia below 0.2 mg/L, and nitrite below 0.02 mg/L. Dissolved oxygen (DO) was maintained at over 99% of the saturation value by adding pure O_2 to the system. A computerized multiprobe Rilheva® system (Xeo4, Italy) was used to continuously monitor DO, pH, and temperature in each tank. The nitrogen and phosphorus forms and chemical oxygen demand (COD) were analyzed using photometric methods (Nanocolor, Macherely-Nagel) after 0.45-μm (pore size) filtration (Schleicher & Schuell) and dilution (1:1 or 1:2) with deionized water, according to APHA Standard Methods (Eaton *et al*., 2005), whereas a single parameter of water quality kit was employed to measure ammonia and nitrite once a day. During the acclimation period, all fish were fed Hendrix-Skretting Power Excel feed for marine fish.

13.7.2.1 Chronic hypoxia and hyperoxia exposure trial

After the acclimation period, 35 fish were transferred into each of three experimental tanks (600 L) connected to the same recirculation system and allowed to acclimate for five days. Then one of the tanks (control) was maintained under normoxic conditions (DO, 8.1±0.3 mg/l, 99-100% saturation), the second one under moderate hypoxic (4.3±0.8 mg/L, 51% of saturation), and the third tank under hyperoxic (DO 13.5±1.2 mg/L, 155% saturation) conditions. The hypoxic DO value was chosen on the basis of our observations on hypoxia tolerance level or oxygen consumption of sea bass and in accordance with experimental protocols used in previous studies (unpublished data) conducted by our group using an identical set up.

The air stones were removed from the second tank, the intake valve was turned down, and water flow adjusted until hypoxic DO levels were

achieved, which occurred within two days; this did not led to significant built-up of excreta compared to controls, so that groups did not differ in more than ambient O_2 levels (pH 7.7-7.9, nitrite 0.01-0.02 mg/L, and ammonia 0.1-0.2 mg/L). Pure O_2 was added to achieve hyperoxic DO levels. At the beginning of the experiment, the mean body weight of the sea bass was 90.1±24.5 g for the control group, 97.6±13.6 g for the 'hypoxia' group, and 95.7 ± 23.8 g for the 'hyperoxia' group. The fish in the three tanks were fed daily at a rate of 1.5% body weight (BW), but after four days of continuous exposure to hypoxia, fish of this group started to consume less than the controls, so we reduced the quantity of feed to 1.0-0.7% BW. At the same time we increased the daily ration for the hyperoxia group to 1.8% BW as they showed a greater appetite. Five fish from each of the three groups were sampled at the start of the experiment (day 0), and then after continuous exposure for 24h, 48h, 5days, and 15days to the respective experimental conditions. In the case of the hypoxic group the time of sampling was calculated without considering the 2-day period of declining DO levels down to 4.3 mg/L. After 15 days of hypoxia and hyperoxia exposure, the DO levels in the two tanks were adjusted back to the normal, saturated levels within 1 h by increasing or decreasing the O_2 addition in the respective tanks and, after a 24-h recovery period, five fish were sampled from each tank. The fish were not fed during this recovery period. All sampled fish were humanely sacrificed by severing the spinal cord and blood supply to the head within a few seconds to minimize handling stress. For the molecular biology analysis, brain, heart, liver, kidney, spleen, and muscle were isolated, frozen immediately in liquid N2, and stored at -80°C.

13.7.2.2 Acute-hypoxia exposure

Five fish from the same, previously described stock were transferred into each of two 100L tanks connected to the recirculation system and after five days of acclimation were exposed to severe hypoxia (1.9±0.2mg/L). The hypoxic DO value was chosen on the basis of our previous observations on sea bass (immediate exposure to 2.0 mg/L was lethal within 4 hours, whereas preconditioning to hypoxia lowered the mortality rate).

The hypoxia level was decreased and maintained in a flow-through tank as described previously. Fish from the first tank were sampled 4 hours after the target level of DO (1.9 mg/L) was achieved, immediately before they were close to death, whereas fish from the second tank were

at the same moment, immediately reoxygenated normally for 24 hours and sampled at the end of the recovery period.

13.7.3 Quantitative Real Time RT-PCR

13.7.3.1 GR and HMGCoAR

We used the $2^{-\Delta\Delta Ct}$ method to analyse the relative changes in the case of GR and HMBCoA gene expression. Total RNA extracted from sea bass tissues was reverse transcribed into cDNA using High Capacity cDNA Archive kit (Applied Biosystems). PCR primers for the amplification of a ~100 bp product were designed by Applied Biosystems. Forward and reverse primers were placed in two consecutive exons to reduce the signal from genomic DNA. TaqMan PCR was performed using an ABI prism 7000 Thermocycler Sequence detection system (Applied Biosystems). C_T (cycle threshold) values corresponded to the number of cycles at which the fluorescence emission monitored in real time exceeded the threshold limit. To reduce pipetting errors, master mixes were prepared to set up triplicate reactions for each sample and for every single test sample, a quantitative PCR for both the target and the housekeeping gene was performed.

For the $\Delta\Delta C_T$ calculation to be valid, the amplification efficiencies of the target and reference gene must be approximately equal, as described in the Applied Biosystems User Bulletin No.2 (P/N 4303859). A sensitive method to assess whether two amplicons have the same efficiency is to look at how ΔC_T varies with template dilution. For this, in the case of GR for example, serial dilutions (1.0, 0.5, 0.2, 0.1, 0.05, 0.02 and 0.01) of cDNAs, were amplified by real time PCR using target and housekeeping gene specific primers and the ΔC_T, i.e., $C_{T(GR)} - C_{T(ACTIN)}$, was calculated for each cDNA dilution. Each point of dilution was tested in 3 replicates. The plot of the log cDNA dilution versus ΔC_T (Figure 13.8) confirmed the similar amplification efficiencies as the absolute value of the slope resulted close to zero (0.0329).

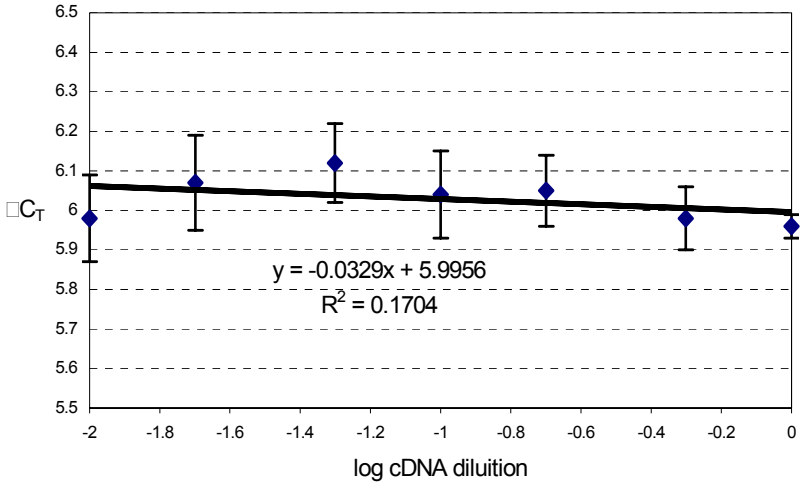

Figure 13.8: Relative efficiency plot. The efficiency of amplification of target gene (GR) and and internal control (β-Actin) was examined using real time PCR and Taqman detection. Serial diluitions of cDNA were amplified by real-time PCR using gene-specific primers.The α C_T (Ct_{GR}-Ct_{Actin}) was calculated for each cDNA diluition. The data were fit using least-squares linear regression analysis (N = 3).

Furthermore, to verify the presence of a single specific amplification product, the primers for GR to be used in real-time experiments were tested in a standard PCR reaction on cDNA from liver tissue. Thermal conditions for this PCR were designed to be very similar to the real-time cycling. The amplification products were then analysed by agarose gel electrophoresis and a single band of the expected size was detected. The accuracy of the amplification was confirmed by cloning and sequencing the gel-excised amplicon. Moreover, as a second sequence for GR was isolated in sea bass (NCBI Genbank Accession no. AY619996), we carefully verified that both primers as well as the Taqman® probe did not present similarities with this sequence. All these points taken together avert the possibility of an unspecific amplification with our real-time experiments.

13.7.3.2 HIF-1α

In the case of HIF-1α total RNA extracted from sea bass tissues was subjected to real-time RT-PCR using the absolute mRNA quantification method. The absolute number of HIF-1α transcript copies was quantified by comparing them with a standard graph constructed using the known copy number of mRNAs of this gene. A forward and a reverse primer

were designed based on the mRNA sequences of the *Dicentrarchus labrax* HIF-1α we had identified (accession no. DQ171936), flanking a region from 1- 487 of the HIF-1α coding sequence. This primer pair was used to create templates for the *in vitro* transcription of cRNAs for HIF-1α: The forward primer was engineered to contain a T7 phage polymerase promoter gene sequence to its 5' end and used together with a reverse primer in a conventional RT-PCR of total sea bass liver RNA. RT-PCR products were then evaluated on agarose gel stained with ethidium bromide, cloned, and subsequently sequenced. *In vitro* transcription was performed then, using T7 RNA polymerase.

The molecular weight (MW) of the *in vitro*-transcribed RNA for HIF-1α gene was calculated according to the following standard formula: HIF-1α MW = HIF-1α MW = [129 (no. of A bases) x 329.2) + 69 (no. of U bases) x 306.2) + 66 (no. of C bases) x 305.2) + 98 (no. of G bases) x 345.2)] + 159. The result was 163983.2. Spectrophotometry at 260 nm gave a concentration of 235 ng/μl for HIF-1α. Therefore, the concentration of the final working solution was 8.63E+11 molecules/μl.

The cRNAs produced by *in vitro* transcription were then used as quantitative standards in the analysis of experimental samples. Defined amounts of cRNAs at 10-fold dilutions were subjected in triplicate to real-time PCR using one tube-two step TaqMan RT-PCR. The Ct (cycle threshold) values obtained were then used to create the standard curve. Figure 13.9 represent the standard curve established for the HIF-1α.

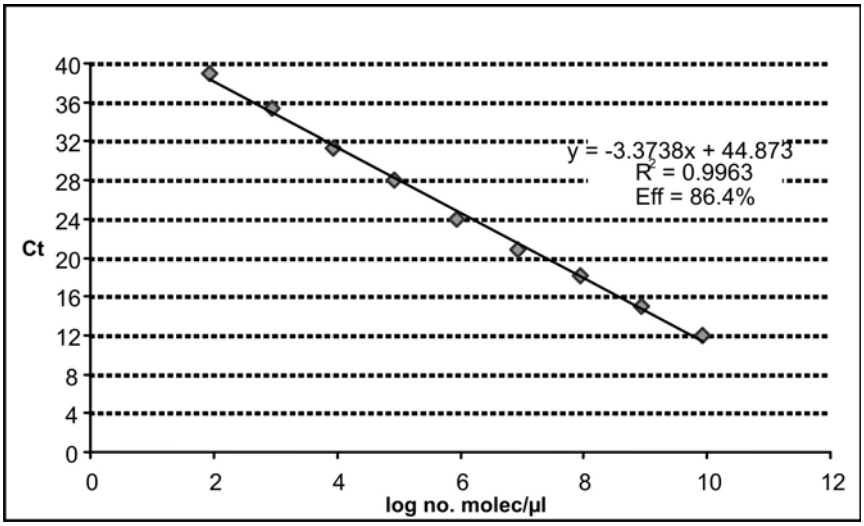

Figure 13.9: Standard curve for HIF-1α obtained by amplification curves of descending 10-fold dilutions of standard cRNAs. Defined amounts of cRNAs were subjected in triplicate to real-time PCR using one-step TaqMan technology.

For the quantitation of HIF-1α gene transcripts an aliquot of total RNA extracted from the experimental samples was subjected, in parallel to standard cRNAs of HIF-1α, to real-time PCR under the same experimental conditions as used to establish the standard curves. Real-time Assays-by-Design^{SM} PCR primers and gene-specific fluorogenic probes were designed by Applied Biosystems (ABI). TaqMan® PCR was performed on an ABI Thermocycler too. Data from the Taqman® PCR runs were collected with ABI's Sequence Detector Program. The data were statistically compared using one-way analysis of variance (ANOVA). The level of statistical significance was set at $P < 0.05$.

13.7.4 Effect of High Rearing Density Stress Conditions on Cortisol and Gene Expression

13.7.4.1 Cortisol

Levels of cortisol in fish reared at densities of 80 and 100 kg/m^3 were significantly higher (124±12 and 280±50 ng/ml, respectively) than those of the group reared at 10 kg/m^3 (60±25 ng/ml).

13.7.4.2 Glucocorticoid receptor

Density stress significantly reduced GR mRNA abundance in the groups of sea bass reared at 80 and 100 kg/m^3 (Figure 13.10). The levels of mRNA were fourfold higher in the control group than in the group reared at 100 kg/m^3. There were no significant differences in the levels of GR mRNA between the groups reared at 10 kg/m^3 and 50 kg/m^3. These data support a downregulation of GR by the cortisol. GR mRNA levels decreased inversely with blood cortisol levels in the groups reared at high population density (Terova *et al*., 2005).

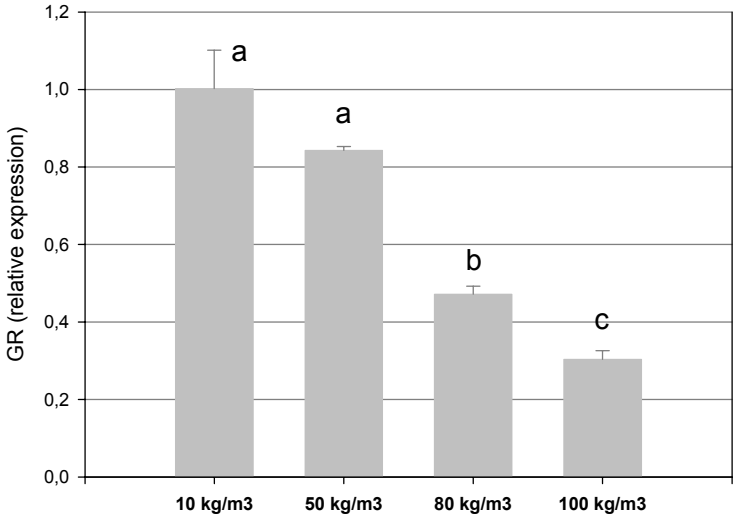

Figure 13.10: Glucocorticoid receptor expression levels measured by real-time PCR in D. labrax reared at 10, 50, 80 and 100 Kg/m^3 density. Cytoskeletal actin has been used as endogenous control. The means of three animals in each group are shown. Bars indicate standard error of the mean. Differences were determined by unpaired t-test di Student. Different letters indicate significantly different means ($P < 0.05$).

In accordance with our data, several other studies support downregulation of GR by cortisol in fish (Maule and Schreck, 1991; Pottinger, 1990; Shrimpton and Randall, 1994). GR concentrations in gills decreased inversely with blood cortisol levels in *Oncorhynchus kisutch* (Shrimpton, 1996) and *Salmo salar* (Shrimpton and McCormick, 1998). Recently, Sathiyaa and Vijayan (2003) also showed that the abundance of GR mRNA induced by cortisol was upregulated in trout hepatocytes *in vitro*, but this result remains to be verified *in vivo*.

13.7.4.3 HMGCoA, HSP70 and HSP90

Density stress significantly increased HMGCoAR mRNA only in the group of sea bass reared at 100 Kg/m^3 (Figure 13.11). The levels of mRNA were eleven fold higher in the group reared at 100 kg/m^3 than in the control group. There were no significant differences in the levels of HMGCoAR mRNA abundance between the groups reared at 10, 50 and 80 kg/m^3.

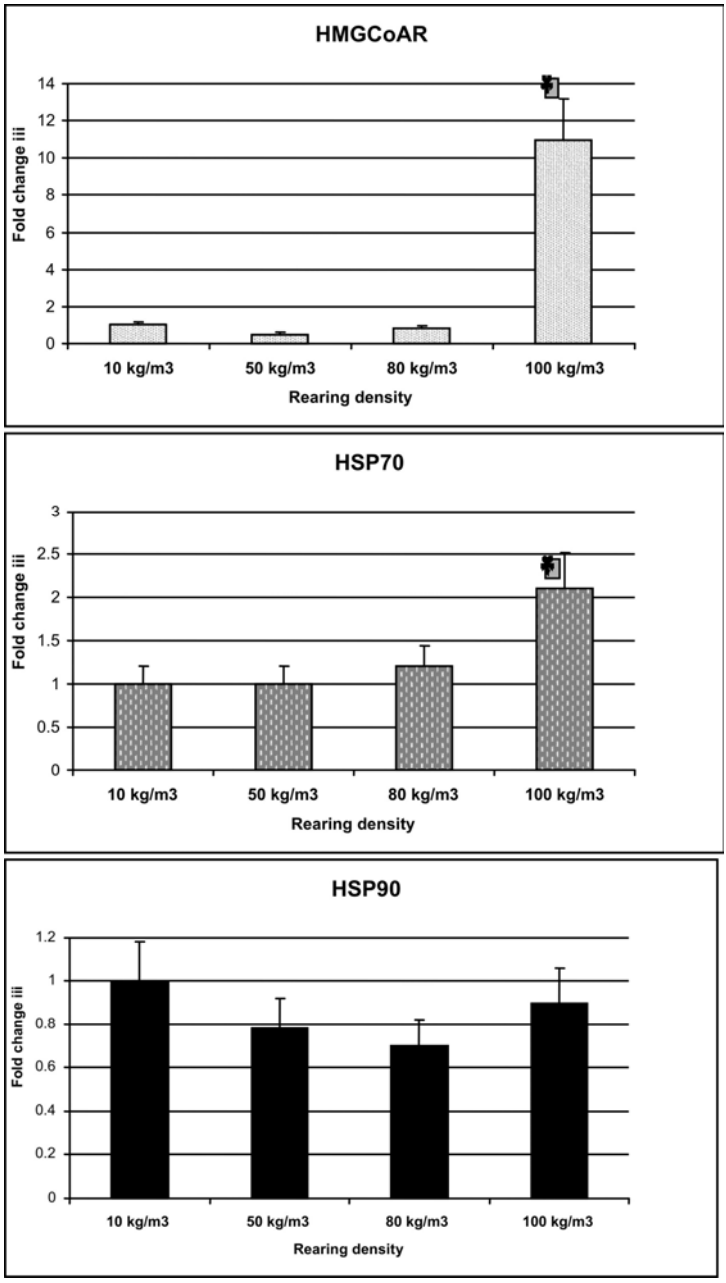

Figure 13.11: Real time RT-PCR quantification of HMGCoAR, HSP 70 and HSP 90 mRNA abundance in the liver of Dicentrarchus labrax reared at 10, 50, 80 and 100 Kg/m^3. Cytoskeletal actin has been used as endogenous control. The means of three animals in each group are shown. Bars indicate standard error of the mean. Differences were determined by unpaired t-test di Student. (*) indicates significantly different means from the control group ($P < 0.05$).

We tested also the mRNA abundance of sea bass heat shock protein 70 (HSP70) (accession no. AY423555) and HSP90 (accession no. AY395632) previously isolated by our group (Gornati *et al*., 2004). HSPs are a wide family of conserved stress-related proteins, classified according to their molecular weight, present in all organisms including fish (Basu *et al*., 2002). The abundance of HSP70 mRNA was twofold higher in the group reared at 100 kg/m^3 than in the control group (Figure 13.11), whereas there were no significant differences in the abundance of the same between the groups reared at 10, 50 and 80 kg/m^3. The abundance of liver HSP90 mRNA levels were similar between the groups reared at 10, 50, 80 and 100 kg/m^3 (Figure 13.11). The response of this gene seems to be less affected by density stress.

13.7.5 Effect of Hypoxia Stress Conditions on Gene Expression

13.7.5.1 HIF-1α

A considerable amount of data is available on HIF-1αs mRNA transcript regulation in a broad range of mammalian tissues (Wiener *et al*., 1996; Rossignol *et al*., 2002; Zhao *et al*., 2004; Wang *et al*., 2006). In contrast, although recent technological developments have made it possible to measure patterns of gene expression, only few published reports are available on tissue expression patterns of HIF-1α in fish exposed to hypoxia. The few data that do exist suggest that HIF-1α subunits are expressed in many fish tissues, such as liver, gonad, and embryonic tissues (Soitamo *et al*., 2001). In Atlantic croaker (*Micropogonias undulatus*), a hypoxia-tolerant marine fish, Rahman and Thomas, (2007) cloned and sequenced two subunits of HIF and investigated the adaptive response of this species to low oxygen levels. Another investigation was done previously by Law *et al*. (2006) in a different hypoxia tolerant species, the freshwater grass carp (*Ctenopharyngodon idellus)*. These studies have made an important contribution to our understanding of the molecular basis of the adaptive response to low oxygen levels in hypoxia tolerant species, whereas studies on hypoxia-sensitive species are still lacking.

Accordingly, we investigated the molecular response of a marine hypoxia-sensitive teleost species such as sea bass. We utilized real-time PCR technology to carefully monitor dynamic changes in levels of HIF-1α mRNA in response to acute and chronic hypoxic stress in this species.

HIF-1α was ubiquitously expressed under both normoxic and hypoxic conditions (Terova *et al*., 2008). The mRNA copy number was higher in the liver of sea bass, with lower expression levels being detected in brain, heart, muscle, and kidney, and negligible expression in spleen (Figure 13.12). Widespread expression of HIF-1α is also known to exist in the grass carp (Law *et al*., 2006); however, in this species another HIF-1α cDNA variant, the 4α was ubiquitously expressed in normoxia, differing from HIF-1α which exhibited a more restricted expression profile. High expression of HIF-1α mRNA was also observed in human organs under non hypoxic conditions (Wiener *et al*., 1996; Rossignol *et al*., 2002).

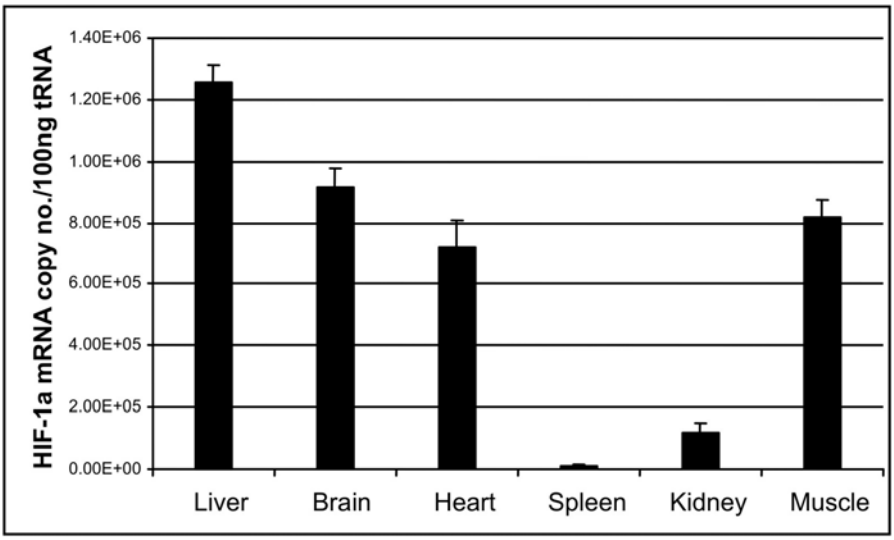

Figure 13.12: Expression pattern of HIF-1α mRNA in sea bass hypoxic tissues as determined by real-time quantitative PCR. Fish were sampled after 15 days of continuous exposure to the chronic hypoxia conditions (DO, 4.3 ± 0.8 mg/L, 51% of saturation). HIF-1α copy number was normalized as a ratio to 100 ng total RNA. The means of five animals in each group are shown. Bars indicate standard error of the mean.

Acute-term exposure to severe hypoxic conditions (4 hrs) and chronic-term exposure to moderate hypoxia (15 days) promoted a remarkable increase in the transcript levels of HIF-1α mRNA, which significantly exceeded the levels of the controls (Figure 13.13 and 13.14 respectively). HIF-1α mRNA levels increased threefold after 4 hrs of exposure to severe hypoxia and three-, seven-, and tenfold after 48 h, 5, and 15 days, respectively, of moderate hypoxia exposure. The expression

patterns were then rapidly reversed upon reexposure to normoxia. These data could suggest that HIF-1α is involved in the early phase of an adaptation response to hypoxia in sea bass. In addition to affecting protein, hypoxia seems to regulate the HIF-1α response at the mRNA level in grass carp, too. In this hypoxia-tolerant species HIF-1α mRNA was upregulated in the kidney after short-term (4 hours) hypoxia exposure and not in any tissue after long-term (4 days) exposure, whereas HIF-4α mRNA was upregulated in several tissues after both short- and long-term hypoxia exposure (Law *et al*., 2006). In another hypoxia-tolerant species, Atlantic croaker (*Micropogonias undulatus*), the expression of HIF-1α and HIF-2α mRNAs, which induction is similar after long-term (3 weeks) hypoxia exposure, is regulated differently during short-term hypoxia. The HIF-1α mRNA level increased after 3 days of hypoxia exposure, whereas HIF-2a mRNA levels did not change significantly.

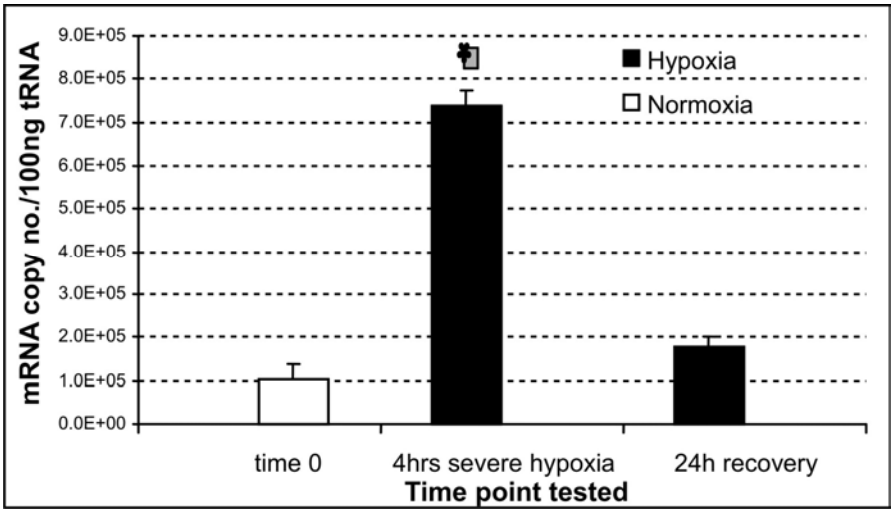

Figure 13.13: Expression levels of HIF-1α measured by real-time PCR in D. labrax liver in the course of the acute hypoxia exposure. HIF-1α mRNA copy number was normalized as a ratio to 100 ng total RNA. Fish were sampled after continuous exposure for 4hrs, to severe hypoxia conditions (DO, 1.9±0.2 mg/L). After 4 hrs of hypoxia the DO levels were adjusted back to normoxia levels and fish were sampled after 24 h of recovery. The means of five animals in each group are shown. Bars indicate standard error of the mean. Differences were determined by one-way analysis of variance (ANOVA). (*) indicate significantly different means from controls, for each time point tested ($P < 0.05$).

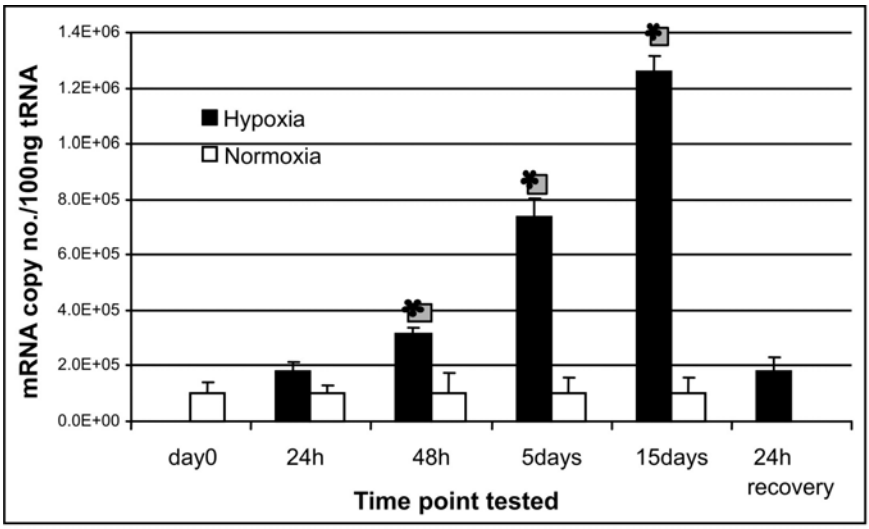

Figure 13.14: Expression levels of HIF-1α measured by real-time PCR in D. labrax liver in the course of the chronic hypoxia exposure (DO, 4.3±0.8 mg/L, 51% of saturation). HIF-1α mRNA copy number was normalized as a ratio to 100 ng total RNA. Fish were sampled at the start of the experiment (day 0), and then after continuous exposure for 24h, 48h, 5, and 15days to the hypoxia conditions. After 15 days of hypoxia the DO levels were adjusted back to normoxia levels and fish were sampled after 24 h of recovery. The means of five animals in each group are shown. Bars indicate standard error of the mean. Differences were determined by one-way analysis of variance (ANOVA). (*) indicate significantly different means from controls, for each time point tested ($P < 0.05$).

It would be interesting to determine whether HIF-1α and HIF-2α have different roles in sea bass, too, during adaptation to hypoxia as indicated in several recent reports (Compernolle *et al*., 2002; Hu *et al*., 2003) and this will be the goal of our future investigations. A long hypoxic period, as in our study, did not require long periods of time for the HIF-1α transcripts to return to 'normality'. In fact, the levels of mRNA reached those of the controls after 24 hours of water reoxygenation. A similarly rapid recovery was also seen after acute hypoxic stress. Therefore, it seems that the extent to which hypoxically stressed fish display high levels of HIF-1α expression upon return to adequate oxygenation condition is not related to the severity of the hypoxic stress sustained by the animals.

The dissolved O_2 is also a known and predictable factor that influences feed intake, which is taken into account in farming condition (Alanärä *et al*., 2001). Fish are dependent on a sufficiency of dissolved

oxygen, and reductions in O_2 concentrations may lead to decreased feed intake, as it was observed also in the course of our chronic hypoxia exposure trial. However, the observed effects in HIF-1α gene expression reflect oxygen differences, rather than nutritional differences, as HIF-1α mRNA levels were significantly increased, as compared with control levels, after 48 hours of hypoxia exposure, whereas differences in feed intake were observed after some days of exposure.

13.8 CONCLUSION

In conclusion, we have isolated the complete coding sequences for GR, HMGCoAR and HIF-1α in sea bass (*Dicentrarchus labrax*). The nucleic and amino acid sequences of all are highly homologous to the previously identified teleost, avian and mammalian respective orthologs. A high rearing density stress affects GR mRNA, whose abundance in the liver decreased inversely with blood cortisol levels. The same type of stress influences also HMGCoAR transcriptional activity, increasing its mRNA abundance in the liver. Therefore, GR and HMGCoAR may be valid bioindicators capable of providing useful information about fish welfare. Acute- and chronic-term hypoxia induces up-regulations in the expression of HIF-1α gene, and these expression patterns are rapidly reversed upon reexposure to normoxia. Future studies are necessary, however, to completely elucidate the underlying mechanism of HIF-1α activation in marine hypoxia-sensitive species.

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14

Diversity and Distribution of Fish Genetic Resources in Anamalai Hills of Western Ghats, India

ARUNACHALAM MANIMEKALAN AND DEVARAJAN THANGADURAI

14.1 INTRODUCTION

In India, the traditional approach to wildlife conservation has been focused on the 'charismatic' large vertebrates and their habitats. This has involved the creation of sanctuaries and national parks for their protection. The Anamalai Wildlife Sanctuary is no exception. Since the Anamalai Wildlife Sanctuary contains some threatened and endemic species of fishes, there is an urgent need to consider their conservation. Many of these species are under tremendous stress due to habitat changes and other human induced impacts. There has been no systematic effort to assess the status of such fishes, their distribution and ecological requirements other than baseline inventorisation. However such information is not sufficient to prepare specific conservation strategies. The aim of the present research proposal is therefore to provide information on the ecology and biodiversity of the fishes in relation to species richness and abundance, assess habitat changes, their causes and evolve strategies for their conservation. This study will be conducted in Anamalai Wildlife Sanctuary which is spread over an area of 958 km^2. This sanctuary can be reached through Topslip in Anamalai hills on the Western Ghats, India. The average elevation ranges between 900-1200m, with the highest peak being Anamudi, situated in the Anamalai hills at 2965m. An ecological paradise, this sanctuary encompasses a National

Park with an area of 108 sq km^2. About 800 out of 2000 species of southern Indian flora are distributed here. This sanctuary nurtures many arboreal animals, ground animals and avifauna.

Fishes constitute approximately half of all described vertebrates, (24,618 species out of the total of 48,170) and comprise of 482 families with living species. Eight largest families, each with over 400+ species contain approximately 33% of all species (some 8,039), which are in descending order, Cyprinidae, Gobidae, Cichlidae, Characidae, Loricariidae, Labridae, Balitoridae and Serranidae. Approximately 100 new species of freshwater fishes are described each year, compared with two new bird species (Maitland, 1995).

India is endowed with a vast network of rivers, canals, reservoirs, tanks and ponds and backwaters, which harbour a rich and diverse fish fauna with nearly 11% of the total fish species of the world. About 2,200 species are known from the Indian waters of which 73 live in upland coldwater, 544 inhabit warm waters of the plains, 143 live in brackish waters and 1440 in the marine environment.

In India the size of fishes is highly variable from a tiny two cm Horadandichthys to giant 8m sharks. They have elongate (eels) to balloon shaped bodies (puffer fish). Some species are beautifully coloured (barbs) while others are drab (catfishes). Some are fast and graceful while others are sedentary. They live in almost every conceivable type of aquatic habitat. They are found in the coldwaters of the Himalayan streams to the near boiling hot springs of Vaitarna river; hillstream waters to saline lakes of Rajasthan. They are found in the highly torrential streams of the Western Ghats (*Travancoria jonesi*) to the total darkness in the sub terranean waters in Kerala (*Horaglanis krishnai*) with no eyes and skin pigments. Many fishes have developed special air-breathing organs, which help them to live in stagnant swamps, while others living in fast flowing streams have developed organs for attachment to the substratum.

Threats to fishes that can render species vulnerable or endangered can arise in several different ways, most, but not all of them are man-made. Habitat alteration or destruction, pollution, over-exploitation and incidental or intentional introductions of species or genetic strains are the principal threats to rare and sensitive species. The most diverse and complex ecosystems, which are usually those with the richest faunas, are likely to be those that are most sensitive to perturbation (May, 1975). To date, fish conservation has been concerned with endangered and threatened species, but the conservation of communities of fishes, their habitats and the whole of the ecosystems should be a long-term aim.

Conservation of aquatic systems is essential for the maintenance of a global environment suitable for man and the biosphere as it is now (Lovelock, 1987). Fish conservation is essential, as fishes are valuable to mankind in several aspects.

Fishes are used as indicators of pollution; the presence of a large number and variety of fishes in a river is a good indication that the water is unpolluted and suitable for extraction and use for humans. Fish in laboratories can be used for bioassay studies and even to set off alarms when water supplies suddenly become polluted. Ecologically, fishes help maintain the ecological balance in the aquatic system. In many ways fishes provide practical and material benefits to man. So the understading of the fish diversity and distribution of a particular area is very important for its conservation.

The objectives of this present study are to study the diversity, abundance and distribution of the fishes; to study the current conservation status of the fishes; and to develop guidelines for the better management of endemic and threatened fishes.

14.2 STUDY AREA

14.2.1 Geography and Climate

The Indira Gandhi (formerly Anamalai) Wildlife Sanctuary (10°12' and 10°54' N and 76°44' and 77°48' E) in Tamil Nadu is one of the largest sanctuaries in southern India (Figure 14.1). Created in 1976, it covers an area of about 987 km^2. It is located mainly in the Valparai Taluk, but extends to Pollachi and Udumalpet Taluks of Coimbatore district and Kodaikanal Taluk of Dindugal district. It extends 45 km north-south, and 25 km east-west. It is about 90 km from Coimbatore city. Three major public roads from Pollachi town pass through the Sanctuary - the Pollachi-Chalakudi road through Valparai, the Pollachi-Parambikulam road through Topslip and the Pollachi-Munnar road through Udumalpet range.

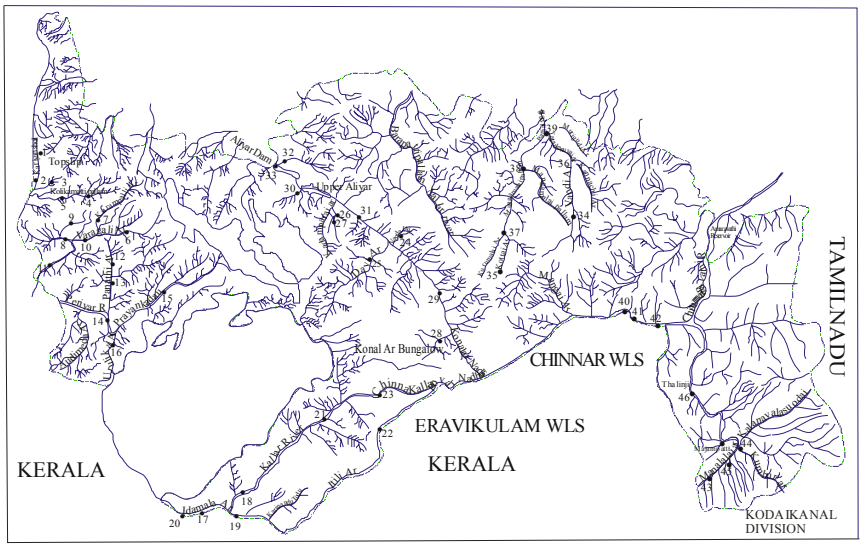


Figure 1: Sampling Sites and Streams & Rivers of IGWLS & National Park

In what can be termed the core area of the sanctuary, there exists nearly 180 km^2 of tea and coffee estates that are under private ownership; Valparai is the closest town. The Sanctuary is bordered in the south-west by Parambikulam Wildlife Sanctuary (287 km^2), in the south by the Reserve Forest of Chalakudi Forest Division and Eravikulam National Park (97 km^2), in south-east by Chinnar Wildlife Sanctuary (90 km^2) all in Kerala State, and in the east mostly by the cultivated plains. These sanctuaries along with the Reserve Forest of Nelliyampathi Hills form a large conservation area for large and wide ranging species such as elephant, gaur, and tiger.

Table 14.1: Habitat characteritics of 46 study sites

S. No.	Study site	Altitude	Forest type	Stream order	Stream Width	S. Depth	Area	Volume	Mean Velocity	Turbidity	Disturbance
I Parambikulam river basin											
1	Karian shola cheak Dam	740	Ever green	3	5	0.3	1.5	150	Stagnent	40	40
2	Erumaparai stream	720	Deciduous	3	4	1	4	400	Stagnent	60	60
3	Ambili cheak Dam	750	Ever green	2	15	1.2	18	1800	Stagnent	40	80
4	Kolikamuthi pallam Elephant camp	685	Teak plantation	3	8	1.1	8.8	880	Stagnent	40	80
5	Kolikamuthi pallam	680	Teak plantation	3	15	1.5	22.5	2250	Stagnent	30	100
6	Varakaliyar shoal	650	Ever green	4	7	0.75	5.25	525	Low	0	30
7	Koorampalli Ar	640	Ever green	3	2.5	0.8	2	200	Low	0	20
8	Chinnar near Peryamula parai	620	Teak plantation	4	55	2.5	137.5	13750	Moderate	60	30
9	Meenmettu pallam	670	Teak plantation	4	15	0.5	7.5	750	Low	20	20
10	Varakali Ar Camp site	640	Teak plantation	4	4	0.5	2	200	Low	0	50
11	Varakaliyar Dam site	600	Teak plantation	4	4	12	48	4800	Low	20	40
12	Panathiyar	650	Ever green	3	3.5	0.9	3.15	315	Low	0	20
13	Karuneer pallam	640	Ever green	3	3	1.2	3.6	360	High	0	5
14	Meen parai	630	Moist	4	7	1.2	8.4	840	High	10	20

			deciduous								
15	Paraiyankadau (Kumiti parai)	670	Ever green	4	10	2	20	2000	Moderate	0	5
16	Urilikal Ar	720	Moist deciduous	3	7	1.5	10.5	1050	High	0	0
II Idamala Ar Basin											
17	Paraman Kadau	420	Reed break	4	25	5	125	12500	Moderate	0	70
18	Kallar	460	Reed break	3	7	2	14	1400	Moderate	0	70
19	Ittli Ar	460	Reed break	4	11	2.5	27.5	2750	High	0	60
20	Itamalai Ar	460	Reed break	4	22	2.5	55	5500	High	0	40
21	Periya Kallar	630	Ever green	4	2.5	1.8	4.5	450	Moderate	10	30
22	Poovar	920	Ever green	3	15	2	30	3000	Moderate	10	20
23	Chinna Kallar	930	Tea plantation	3	19	1.2	22.8	2280	High	0	30
III Aliyar Basin											
24	Vandal Ar	700	Moist Deciduous	3	6	0.8	4.8	480	Low	0	40
25	Devi Ar	690	Moist Deciduous	2	5	0.5	2.5	250	Low	0	20
26	Pannikuli Ar	620	Plantation	2	5	1.2	6	600	Moderate	0	60
27	Pambar	630	Plantation	3	5	1.2	6	600	Low	0	20
28	Konal Ar (near Bungalow)	2155	Grass land Shola	3	3	1.2	3.6	360	Low	0	30
29	Konal Ar	2150	Grass land Shola	3	3	1.1	3.3	330	Low	0	30

30	Lower Poonachi	430	Dry decidous	3	15	0.9	13.5	1350	Moderate	10	40
31	Kadamparai Ar	580	Dry decidous	4	11	1.9	20.9	2090	Moderate	5	30
32	Navamalai Ar	270	Dry decidous	4	7.2	1.7	12.24	1224	0	0	40
33	Aliyar Dam	260	Dry decidous	4	11	2.2	24.2	2420	Moderate	0	20
IV Thirumurthi river basin											
34	Confluence of Vandi Ar & Vendaiyathar	480	Dry deciduous	4	3	0.75	2.25	225	Low	10	40
35	Periyathorai Ar	480	Dry deciduous	4	6	1.6	9.6	960	Moderate	5	20
36	Dattankanavai Ar	460	Dry deciduous	3	11	0.9	9.9	990	Low	0	40
37	Sambathurai Ar	420	Dry deciduous	3	9	1.1	9.9	990	Low	0	30
38	Thoni Ar	400	Dry deciduous	4	9	1.2	10.8	1080	Moderate	5	20
39	Panjalingam Ar (Bellow the Falls)	360	Dry deciduous	4	14	1.2	16.8	1680	Low	5	10
V Amaravathi river Basin											
40	Chinnar (Kattal Parai Ammam paguthi)	420	Moist decidious	4	15	1.7	25.5	2550	High	0	20
41	Manal medu	790	Moist	3	17	1.7	28.9	2890	High	0	10

			decidious								
42	Kopparai kajam (Manjampatti)	780	Moist decidious	3	5	1.8	9	900	High	5	30
43	Manal Allai (Manjampatti)	780	Ever Green	3	8	2	16	1600	High	5	30
44	Kumbur Ar	700	Ever Green	4	4.4	1.2	5.28	528	Moderate	5	20
45	Azhanthoni	700	Ever Green	4	12	2.2	26.4	2640	High	10	20
46	Sallimuthan parai Ar	660	Ever Green	4	9	1.7	15.3	1530	High	10	10

The altitude of the Sanctuary ranges from 220 m in the plains at the foot hills in the east to 2,513 m top Thanakkanmalai in the Grass Hills. Hilly tracts form over 90% of the total area, extending north-west to south-east with an elevation from 700 m at Topslip to 2,513 m at Thanakkanmalai. In the north, hills descend precipitously to the cultivated plains. The central portion around the Valparai Town, at an elevation of 900 m to 1,500 m, has been converted to tea and coffee plantations. In the south and south-east parts in the Udumalpet and Amaravathi ranges, the hills are elevated, steep and abruptly descend down to the plains.

The soil found in the Anamalai Hills is classified as lateritic soil (Krishnan, 1982). It is a porous, pitted, clay-like rock with red, brown, grey and mottled colours depending on the composition. The rocks found here are classified into metamorphic and igneous types, and are estimated to be from the Pre-Cambrian period.

Rainfall varies considerably, ranging from 500 mm in the eastern slopes of the Sanctuary to 5,000 mm in the western slopes. The Sanctuary receives both south-west (June to September) and north-east (October and November) monsoons, with about 80% of the rainfall being during the former. The day time temperature varies considerably from 23°C to 40°C at the foot hills (200 to 350 m) to 20°C to 30°C at higher elevations (1,800 to 2,300 m). In the night, it ranges from 15°C to 25°C at the foot hills and from 10°C to 20°C at medium elevation of 900 m to 1,200 m. The temperature is lower at higher elevations, going down to 0°C in December and January at about 2,000 m. March to May are the hottest months (Kumar *et al.*, 1998).

According to rainfall and mean temperature for each month, we identified three distinct seasons; 1) Dry: low temperature and no or less rainfall (January to April); 2) First wet (South-west monsoon): moderate temperature and high rainfall (May to August); and 3) Second wet: moderate temperature and moderate rainfall (September to December).

14.2.2 History

The Anamalai (Elephant) Hills have been named after the elephants which were once found in abundance there. It is a continuation of the Western Ghats immediately south of the Palghat Gap. In the early days of British rule, the forests in this area did not have any specific management nor were any of the forests properly surveyed. After the Bombay dockyard started construction of vessels of war (Sundararaju, 1987), teak trees were required in large quantities. This demand was met from the forest of Anamalai Hills. British surveyors surveyed Anamalai Hills for the

first time in 1820s and reported surpassing quantity and quality of teak trees. After the survey, the Bombay Marine Company started extraction of timber from this forest between Malabar border and north of Vengoli-Umayamalai ridge. During this time, most of the south division of Coimbatore was over exploited. The major tree species extracted were *Tectona grandis, Dalbergia latifolia, Pterocarpus marsupium, Terminalia tomentosa, T. bellerica, T. arjuna* and T. *indica.* Extraction declined considerably in 1862-63 when the construction of vessels of war in Bombay was given up. Reservation of forest in this tract commenced from 1883, after the enactment of Madras Forest Act in 1882. When the process of reservation was over, the Government had set apart an area of 19,114.13 ha of virgin evergreen forest land in the Valparai area for raising tea, coffee, and cardamom plantations. This land was then leased to private companies for cultivation. The area to be opened up first was the Waterfall Estate in 1890, and was followed by other estates until 1930 (Congreve, 1938). At present 18,032 ha of tea, coffee, and cardamom estates and 3,717 ha of cinchona plantation (half of which have been converted into tea plantation) are situated within the Sanctuary (Sundararaju, 1987). The Forest Department continued logging in the northern side of the Sanctuary till 1976 (Kumar *et al.*, 1998).

14.2.3 Water Resources

A series of reservoirs and weirs have been constructed within and outside the Sanctuary under the Parambikulam-Aliyar Project. Of these, Aliyar, Upper Aliyar, Kadamparai, Upper and Lower Nirar, Thirumurthy and Parambikulam reservoirs come at least partly within the Sanctuary area. The Amaravathi reservoir, the largest in the area, is also partly inside the Sanctuary. These man-made reservoirs are now an important source of water to many animals especially during the summer. A few perennial and many seasonal streams also occur. The perennial rivers are Konalar in the Grass Hills, Varagaliar and Karuneerar in Ulandy range, and Chinnar and Amaravathi rivers in Udumalpet and Amaravathi ranges, respectively (Kumar *et al.*, 1998).

14.2.4 Drainage System

Great importance must be attached to the nature of the watershed in a given place in discussing the distribution of freshwater organisms, especially fishes, as fortuitous dispersal of hill stream fishes through any agency whatsoever must be a very rare occurrence. The streams of

Anamalai hill ranges and the area in the immediate vicinity drain ultimately into three main systems, namely Cauvery the Ponnani and the Periyar.

14.2.5 The Cauvery Drainage

The Cauvery is fed by a number of streams arising in the Central Division of the Western Ghats, viz., the hills of Coorg, Mysore and the Nilgiris. On the east of the Anamalai Hills, a tributary of the Amaravity originates and flows due east and then joins the Amaravity, which in turn joins the river Cauvery further east. The Cauvery thus links up the eastern faces of the Ghats in the Central and the Southern divisions.

14.2.6 The Periyar Drainage

The streams and rivers on the western and south-western faces of the Anamalai and Nelliampathy hills are separated from the water sheds of the north-eastern face by the intervening high hill ranges. The river Chalakudi receives a tributary each from the Anamalai and Nelliampathy hills, flows due south-west and joins the river Periyar close to the sea in Cochin. The Periyar river is fed by a number of streams draining the western face of the Anamalai Hills in the north and the Cardamom Hills in the south. The Periyar watershed though isolated from the north is continuous with the system of waterways further south.

14.3 EARLIER STUDY

14.3.1 Western Ghats

Study on the freshwater fishes of India dates back to 1822 when Hamilton-Buchanan published his account of the fishes found in the river Ganges. Later, Jerdon (1849) published a work on the fishes of South India especially Cauvery river. But a comprehensive and authoritative account on the fishes of India region was published only during 1875-1878 and 1889 by Francis Day. During the intervening hundred years, there was nothing substantially published to fill the lacuna. Publications by Misra (1947, 1976a, 1976b), Jayaram (1981), Datta Munshi and Srivastava (1988), Menon (1987) and Talwar and Jhingran (1991) supplemented information on the freshwater fish fauna of India. In addition, information on the fauna of Western Ghats is also available from the publication by Day (1865).

Hora (1921b, 1937a, 1941, 1942a) added several species from Western Ghats and made surveys in several parts of India. Day (1867a,b) documented the fish fauna of the Nilgiri and Wynaad hills. Bhimachar (1945) made zoogeographical studies of Western Ghats based on the distribution of hill stream fishes. In general, studies on the fish fauna of Western Ghats outside Nilgiri Biosphere Reserve are very few (Jayaram, 1981b; Jayaram *et al*., 1982). Studies on the freshwater fishes of South India started by asistand surgent Frances Day's the monumental classic works, Fishes of Malabar (1865) and Fishes of India (1889). The Travancore region was comparatively well explored (Pillay, 1929; John, 1936; Hora and Law, 1941; Hora and Nair, 1941; Chacko, 1948 and Silas, 1949, 1952). Raj (1941a,b) described two new species from Periyar Tiger Reserve. The Malabar region, especially the region north of Palghat gap still remained underexplored.

Hora's Satpura hypothesis on the distribution of freshwater fishes (1944, 1949) generated interest on the fishes of some rivers of the Western Ghats (Menon 1951, 1955). Bhimachar (1945) described some of the distributional peculiarities of freshwater fishes in the Malabar tract. He subdivided the Western Ghats into a northern section (the Deccan trap area from the river Tapti), a middle section (upto the Nilgiri hills) and a southern section (the Anamalai, Palni and Cardamom hills). The southern (which includes the Anamalai) portions show a high degree of endemism.

14.3.2 Anamalais

Apart from a few basic studies, made, the fishes of Anamalais have not been thoroughly studied. Herre (1942) described *Glyptothorax housei* from Puthutam Estate; Herre and Wash (1945) described *Homaloptera montana* and Silas (1951) studied the fish fauna of Anamalais and Neliampathi ranges and he reported 27 species, in which *Glyptothorax anamalaiensis* is a new description. Perusal of the literature shows that studies are confined to few locations and no comprehensive work was carried out that covered the entire sanctuary. Therefore the aim of the present research is to study the fish population of the sanctuary and to provide information on the current status, ecology, distribution and biodiversity of fish species of Anamalai hills.

14.4 METHODOLOGY

Sampling site was chosen in the streams/rivers to represent a wide range of habitat conditions such as altitude, stream order, habitat types, forest type, velocity, disturbed and undisturbed habitat. At each sampling site, fishes were visually counted as far as possible and then fishes were sampled using gill nets (different 8 mm – 22 mm), cast nets and dip nets depending upon the depth. Fishes were identified according to the keys given by Talwar and Jhingran (1991) and Jayaram (1999); stream order classification based on Horton's (1945) approach, modified by Strahler (1954, 1957) as described in Kuehne (1962). The habitat structure was measured in three dimensions using Gorman and Karr (1978) with modifications based on the Indian conditions with depth, water velocity and the substrate type. Habitat area (m^2) was calculated as the product of the length and mean width of the study reach. Habitat area multiplied by mean depth was used as an indication of habitat volume.

14.5 STATISTICAL ANALYSIS

In order to quantify species diversity for purposes of comparison, a number of indices have been followed. To measure the species diversity (H') the most widely used Shannon index (Shannon and Weaver, 1949), evenness index (E) (Pielou, 1975) and Dominance index (D) (Simpson, 1949) were used. Similarity coefficients of the fish community were calculated by using the widely used Jaccard index (Southwood, 1978). Since the species abundance and their relative frequencies were subjected to cluster analysis, a complete linkage cluster dendrogram was drawn based on Pearson correlation (Lance and Williams, 1967; Dunn and Everitt, 1982). Linear regression analysis was done for species richness and abundance with habitat parameters such as altitude, stream order, water velocity, depth and forest type. The above statistical analysis was performed using window based computer software SPSS.

14.6 RESULTS

14.6.1 Species Diversity, Richness and Abundance

A study was conducted in 46 sites of east and west flowing river systems such as Parambikulam basin, Idamala Ar Basin, Aliyar Basin, Thirumurthi river basin, Amaravathi river Basin of Indiri Gandhi Wild life Sanctuary. A total of 68 species were collected which belonging to 8 orders, 16

families and 30 genera. In this 6 species are found to be new (under publication) (Table 14.2; Figure 14.2). Among the 68 species family belonging to *Cyprinidae* was the dominant family with 44 species (69.9%) compared to other families. The maximum species richness (S), diversity (H') was observed in Parambikulam basin (S-38; H'-1.44) followed by Aliyar (27; 1.35), Idamalai Ar (22; 1.24), Amaravathi (20; 1.30) and Thirumurthi (16; 1.12) river basins (Figure 14.3, 14.4). Amoung the 46 site sampled the maximum diversity and richness was recorded in Varakali Ar Camp site and the maximum abundance was recorded in Ambili check Dam (Figure 14.5-14.7).

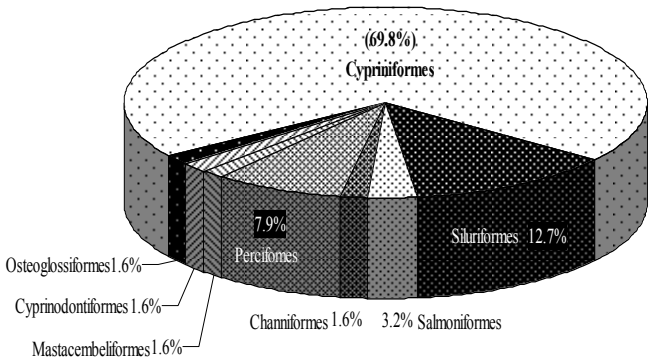


Figure 14.2: Percentage reprecentation of fishes in different order.

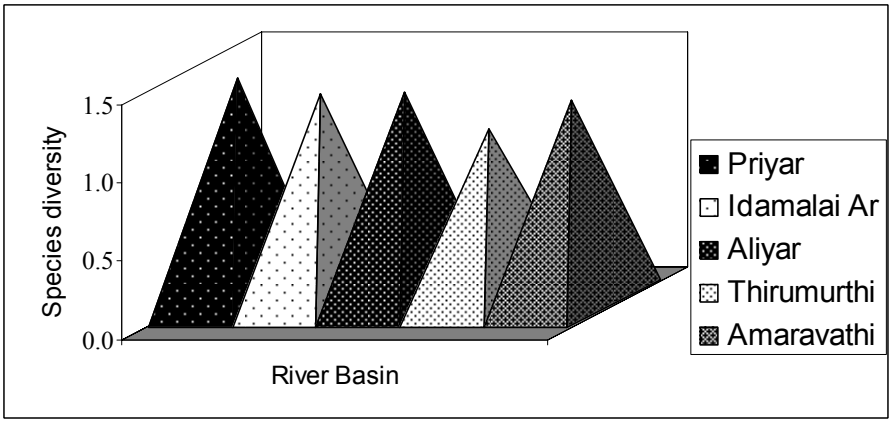


Figure 14.3: Species diversity in different river system in IWLS.

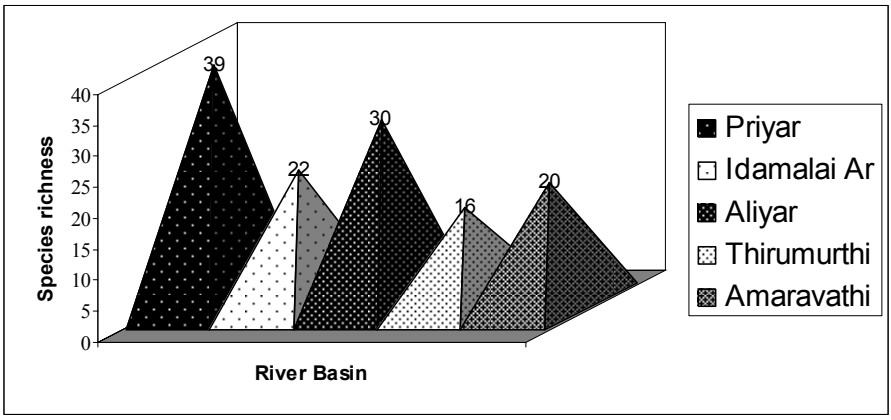


Figure 14.4: Species richness in different river system in IWLS.

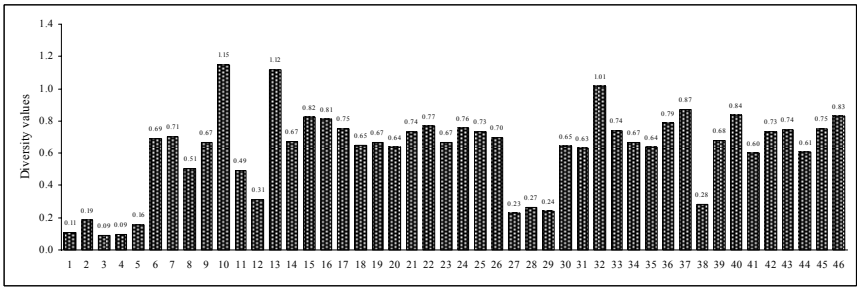


Figure 14.5: Species diversity in amoung 46 sites.

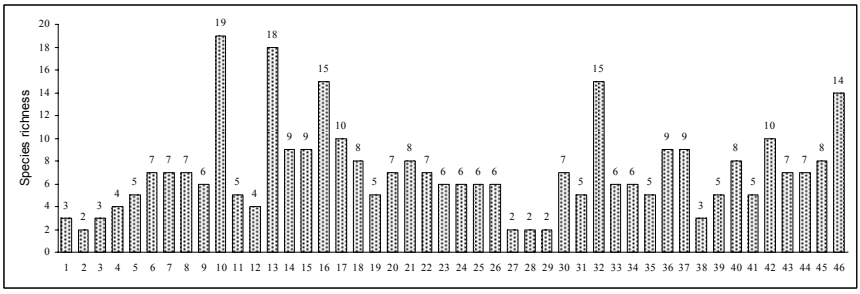


Figure 14.6: Species richness in amoung 46 sites.

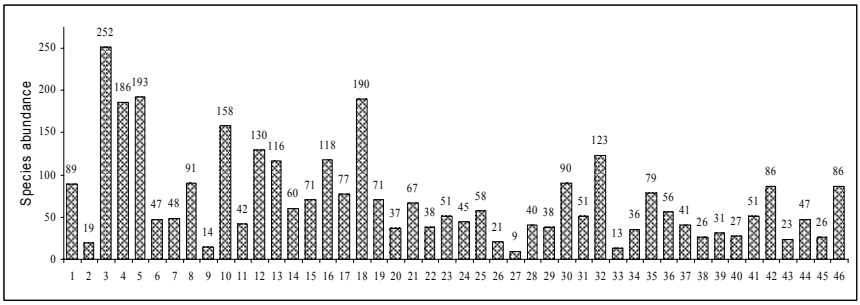


Figure 14.7: Species abundance in amoung 46 sites.

Table 14.2: Species recorded during the present study

S. No.	Species	No. of Location
Order 1 Osteoglossiformes		
Family 1 Notopteroidae		
Genus 1 Notopterus		
1	*Notopterus notopterus* (Pallas)	1
ORDER - ANGUILLIFORMES		
Family ANGUILLIDAE		
Genus Anguilla		
2	*Anguilla bengalensis* (Gray & Hardw.)	1
Order 2 Cypriniformes		
Family 3 Cyprinidae		
Subfamily Cyprininae		
Genus Catla		
3	*Catla catla* (Ham)	2
Genus Cirrhinus		
4	*Cirrhinus mirgala*	1
Genus 2 Cyprinus		
5	*Cyprinus carpio communis* Linnaeus	
Genus 3 Hypselobarbus		
6	*Hypselobarbus dubius* Day	2
7	*Hypselobarbus kolus* (Sykes)	9
Genus 4 Labeo		
8	*Labeo calbasu* (Ham)	1
9	*Labeo rohita* (Ham)	1
Genus 5 Neolissochilus		
10	*Neolissochilus anamalaiensis* sp. nov.	3

11	*Neolissochilus wynaadensis* (Day)	8
Genus 6 Puntius		
12	*Puntius amphibius* (Valencienes)	1
13	*Puntius carnaticus* (Jerdon)	10
14	*Puntius chola* (Hamilton)	1
15	*Puntius fasciatus fasciatus* (Jerdon)	18
16	*Puntius filamentosus* (Valenciennes)	3
17	*Puntius jerdoni* (Day)	1
18	*Puntius melanampyx* (Day)	15
19	*Puntius poovarensis* sp. nov.	1
Genus 7 Tor		
20	*Tor anamalaiensis* sp. nov.	7
21	*Tor khudree* (Sykes)	
22	*Tor khudree malabaricus* Jerdon	
23	*Tor putitora* Hamilton-Buchanan	3
24	*Tor tor* Hamilton-Buchanan	2
Subfamily Cultrinae		
Genus 8 Salmostoma		
25	*Salmostoma boopis* (Day)	2
Subfamily Rasborinae		
Genus 9 Barilius		
26	*Barilius bakeri* Day	2
27	*Barilius barna* Hamilton	8
28	*Barilius canarensis* (Jerdon)	2
29	*Barilius gatensis* (Valenciennes)	19
30	*Babilius kadamparaiensis* sp. nov.	2
Genus 10 Danio		
31	*Danio aequipinnatus* (McClelland)	33
Genus 11 Rasbora		
32	*Parluciosoma daniconius* (Hamilton)	13
33	*Parluciosoma labiosa* (Mukerji)	1
34	*Rasbora* sp.	1
Subfamily Garrinae		
Genus 12 Garra		
35	*Garra gotyla stenorhynchus* (Jerdon)	5
36	*Garra hughi* Silas	14

37	*Garra mcclellandi* (Jerdon)	15
38	*Garra mullya* (Sykes)	33
39	*Garra idamalaiyarensis* sp.nov.	1
Family 4 Balitoridae		
Subfamily Balitorinae		
Genus 13 Balitora		
40	*Balitora brucei* Gray	1
Genus 14 Bhavani		
41	*Bhavania australis* (Jerdon)	1
Genus 15 Homaloptera		
42	*Homaloptera montana* Herre	2
Genus 16 Travancoria		
43	*Travancoria elongata* Pethiyagoda & Kottelat	1
44	*Travancoria jonesi* Hora	1
Subfamily Nemacheilinae		
Genus 17 Nemacheilus		
45	*Nemacheilus denisoni denisoni* Day	4
46	*Nemacheilus guentheri* Day	4
47	*Nemacheilus monilis* Hora	2
48	*Nemacheilus moreh* (Sykes)	2
49	*Nemacheilus ruppelli* (Sykes)	1
Family 5 Cobitidae		
Subfamily Cobitinae		
Genus 18 Lepidocephalus		
50	*Lepidocephalus thermalis* (Valenciennes)	9
Order 3 Siluriformes		
Family 6 Bagridae		
Genus 19 Mystus		
51	*Mystus armatus* (Day)	2
52	*Mystus cavasius* (Hamilton)	3
53	*Mystus montanus* (Jerdon)	1
Family 7 Siluridae		
Genus 20 Ompok		
54	*Ompok bimaculatus* (Bloch)	3
55	*Ompok malabaricus* (Valenciennes)	2
Family 8 Sisoridae		
Genus 21 Glyptothorax		

56	*Glyptothorax housei* Herre	3
Family Clariidae		
Genus 22 Clarias		
57	*Clarias dayi* Hora	1
58	*Clarias dussumieri* Valenciennes	1
Order 4 Salmoniformes		
Family 9 Salmonidae		
Genus 23 Salmo		
59	*Salmo gairdenerii gairdnerii* (Richardson)	2
60	*Salmo trutta fario* Linnaeus	2
Order 5 Cyprinodontiformes		
Family 10 Belonidae		
Genus 24 Xenentodon		
61	*Xenentodon cancila* (Hamilton)	1
Order 6 Perciformes		
Suborder Percoidei		
Family 11 Ambassidae		
Genus 25 Chanda		
62	*Chanda nama* Hamilton	2
63	*Pseudambassis ranga* (Hamilton)	1
Family 12 Nandidae		
Subfamily Pristolepidinae		
Genus 26 Pristolepis		
64	*Pristolepis marginata* Jerdon	8
Family 13 Cichilidae		
Genus 27 Oreochromis		
65	*Oreochromis mossambica* (Peters)	15
Suborder Gobioidei		
Family 14 Gobiidae		
Genus 28 Glossogobius		
66	*Glossogobius giuris* (Hamilton)	1
Order 7 Channiformes		
Family 15 Channidae		
Genus 29 Channa		
67	*Channa orientalis* Bloch & Schneider	1
Order 8 Mastacembeliformes		
Suborder Mastacembeloidei		
Family 16 Mastacembelidae		
Genus 30 Mastacembelus		
68	*Mastacembelus armatus* (Lacepede)	2

Anguilla bengalensis (Gray)

Common name: Indian longfin eel

Vernacular name: Tamil - Vellangoo

Diagnosis: Body elongate; mouth terminal; angle of mouth behind posterior margin of eye; dorsal fin inserted nearer anus than gill-opening; color- yellowish, mottled with dark brown.

Distrubution: Gray (1831) described this species from river Ganges after he described this have been reported from Bengal (McClelland, 1844), Malarar (Day, 1878, 1889) Deolali, Nasik dist. (Hora and Misra, 1938), Poona (Hora, 1942) Bhavani and Moyar rivers and some of their tributaries (Rajan, 1956), Kolhapur dist. Maharashtra (Kalawar and Kelkar, 1956), Bombay (Kulkarni and Ranade, 1974), Silent Valley, Kerala (Rema Devi and Indra, 1986) Moyar (Manimekalan, 2000) Moyar near Kargudi.

Anamalai: Kadamparai Ar

Relative abundance: Not common.

Notopterus notopterus (Pallas)

Common name: Grey featherback

Vernacular name: Mal. - Ambattan vala; Tam. - Vala meen, Chappathi, Ambattan Kathi

Diagnosis: Body oblong and strongly compressed; maxilla extends to middle of eye; preorbital serrated; no transverse bar on back; scales minute; colour - silvery.

Distribution: Pallas (1769) described this species from Indian Ocean (type locality not known) under the genus *Gymnotus*. Later this species kept under the genus Notopterus (Day, 1887, 1889). Day (1887, 1889) described *Notopterus kapirat* and Rahimullah and Das (1936) described *N. osmani* from rivers of Hyderabad, Deccan which is the synonymy of *N. notopterus*. After this species have been reported from Deolali, Nasik dist., (Hora and Misra, 1938, 1976), Poona (Hora, 1942; Tilak and Tiwari, 1976), Coorg dist, Karnataka (Raghnathan, 1989), Ujni wetland. Krishna river, Maharashtra (1990), river Moyar, Nilgiri (Manimekalan, 2000) Aliyar dam site, Throughout India.

Anamalai: Aliyar Dam

Remarks: It is commonly found in tanks, lakes and rivers where it breeds during the monsoon. Since it is a carnivorous fish, its culture has not been attempted on a large scale.

Status: Common.

Cyprinus carpio communis Linnaeus

Common name: Common carp

Diagnosis: Body stout; head triangular; mouth small and oblique; thick lips; 2 pairs of barbels; dorsal fin inserted midway between snout and base of caudal; dorsal spine serrated; scales large; lateral line with 30-40 scales; Color- silvery to golden, fins yellowish to golden.

Distribution: Throughout India

Anamalai: Pannikuli Ar, Pambar

Remarks: The common carp has three recognised varieties: *C. carpio* var. *communis* (scale carp) and *C. c.* var. *specularis* (mirror carp). The mirror carp was brought from Sri Lanka in 1939 and stocked in the Ooty Lake (Tamil Nadu) and introduced in almost all the dam for culture purpose. This fish is an omnivorous bottom dweller.

Relative abundance: Not common.

Hypselobarbus dubius (Day)

Common name: Nilgiri barb

Vernacular name: Tam. - Kozhimeen

Diagnosis: Robust body; no colour bands or spots; 2 pairs of barbels; lateral line complete with 42-45 scales; scales tinged at base.

Distribution: Day (1867) described this species Bhavani river at base of Nilgiris (Gunther, 1868; Day, 1878; Day, 1889). After Day described Hora (1837) reported this species from Cauvery river, Coorg; Raj (1941) reported from Bhavani river, Cauvery river at Coorg, Cauvery river at Mettur dam; Rajan (1955, 1963) reported this species from Bhavani and Moyar rivers, Nilgiri Hills; Sreenivasan (1976) reported from Stanley reservoir, Cauvery river; Jayaram *et al*. (1982) reported from Cauvery river, S. India; Daniels (1993) reported from Nilgiri Biosphere Reserve. In the present study this species was collected from Bhavani at Thavalam and Moyar at Thengumarada, Confluence of Sigur and Moyar river and the confluence of Kakkanhalla and Moyar river. All the study shows the distribution form Nilgiri and Cauvery basin. This is the first record from Anamalais.

Anamalai: Karuneer pallam, Meen parai

Remarks: It forms an important fishery in the Cauvery system. It is found more commonly in hilly regions than in the plains.

Status: Common.

Hypselobarbus kolus (Sykes)

Common name: Kolus

Vernacular name: Koli meen

Diagnosis: Large sized fish with deep body; no colour bands; 2 barbels; dorsal ray weakly osseous, articulated; lateral line complete with 40-43 scales; snout conical with tubercles; eyes large, its upper edge near the dorsal profile.

Distribution: Godavari, Krishna and Cauvery river systems

Anamalai: Chinnar near Peryamula parai, Varakali Ar Camp site, Varakaliyar Dam site, Karuneer pallam, Meen parai, Paraiyan kadau (Kumiti parai), Urilikal Ar, Periya Kallar, Dattankanavai Ar

Remarks: Grows upto a length of 300mm. The adult males possess horny tubercles or 'pearl organs' on the sides of the snout. It forms a good fishery resource.

Status: Not found in the Pune waterways but small populations present in the Krishna river near Satara (Maharashtra). Vulnerable, needs monitoring.

Neolissochilus wynaadensis (Day)

Vernacular name: Tam

Diagnosis: Body elongate; mouth rounded with lower labial fold interrupted; 4 barbels; lateral line 26 to 28 scales; lateral transverse scales 4-6; predorsal scales 10; dark band running from behind the eye to the middle of base of caudal fin; belly light orange in colour.

Distribution: Rainboth (1985) created the genus Neolissochilus from *Acrossocheilus*. Day described N*eolissochilus wynaadensis from* Wynaad and Vythiri, Kerala (Day, 1873; Day, 1878; Day, 1889; Jayaram, 1982; Daniels, 1993; Easa and Basha, 1995). Manimekalan (2000) reported this species from Nilpuzha, Vithiri and Kallar. Earlier workers on Bhavani have not reported this species (Mukerji, 1931; Easa and Basha, 1995). The present study shows the range of extension to Anamalai hills.

Anamalai: Karuneer pallam, Navamalai Ar, Chinnar (Kattal Parai Ammam paguthi), Kopparai kajam (Manjampatti), Manal Allai (Manjampatti), Kumbur Ar, Azhanthoni, Sallimuthan parai Ar

Remarks: It grows upto 250mm in length. It does not form any major fishery in the area.

Status: Endemic.

Puntius amphibius (Valenciennes)

Common name: Scarlet - banded barb

Vernacular name: Tam. - Kulla Kendai

Diagnosis: Spindle shaped body; one pair thin barbels; dorsal ray feebly osseous; lateral line complete with 23-24 scales; predorsal scales 7 or 8; a conspicuous dark spot at base of caudal fin.

Earlier records: Valenciennes (1842) describer this species from Bombay after this described this species have bee report from Malabar and other part of South India (Day, 1865, 1870, 1878, 1889, Madras (Raj, 1916), Poona (Hora and Misra, 1937), Deolali, (Hora and Misra, 1938), Manimala river, Peruntenaruni, Kallada river, Kulathupuzha, Chittar, Central Kerala (Hora and Law, 1941, Karnataka (Hora, 1942), Valparai, Ponnani drainage, Kerala), Headwaters of Cauvery, Coorg (Silas, 1951), Tambraparni River (Silas, 1953), Bhavani and Moyar river, Nilgiri Hills (Rajan, 1956), Kolhapur dist. Maharashtra (Kalawar and Kelkar, 1956), Bhadra river at Bhadravati (David, 1956), Ponda, Colva road, Goa (Tilak, 1973), Cardamon hills (Jayaram *et al*., 1976), Indrayani river, Poona dist. Maharashtra (Yazdani and Mahabal, 1976), Cauvery river (Jayaram *et al*., 1982).

Range: Peninsular India, upto Orissa, Madhya Pradesh and Rajasthan

Anamalai: Confluence of Vandi Ar and Vendaiyathar

Remarks: Grows upto a length of 90 mm. It is a common species in South India and is a potential larvicide fish.

Status: Common.

Puntius carnaticus (Jerdon)

Common name: Carnatic Carp

Vernacular name: Tam. - Pulli kendai, Poaree candee, Saal kendai

Diagnosis: Deep bodied; no colour bands or spots; 4 barbels; dorsal ray osseous, strong and smooth; lateral line curved, complete, with 28-31 scales; predorsal scales 10-12; dark olive green colour on back, dull white on abdomen and sides; fins dusky, outer margin of dorsal fin black. `V' shaped marking on the caudal.

Earlier records: Jerdon (1849) described this species from Cauvery river. The species have been reported from Cauvery (Gunther, 1868), Malabar and Wynaad (Day, 1878, 1889), Bombay (Annandale, 1919), Nilgiris, Wynaad, Mysore and S. Canara), Valparai, Vannamadi (Hora, 1942), bridge, Ponnani drainage (Silas, 1951) Bhavani and Moyar rivers, Nilgiri Hills (Rajan, 1956; Manimekalan, 1998), Moyar river, Nilgiri Hills (Rajan, 1963), Stanley and Tirumoorth reservoirs, Cauvery river system (Sreenivasan, 1976) Cauvery river, S.India (Jayaram *et al*., 1982).

Anamalai: Varakaliyar Dam site, Karuneer pallamMeen parai, Periya Kallar, Pambar, Navamalai Ar, Aliyar Dam, Dattankanavai Ar, Panjalingam Ar (Bellow the Falls), Sallimuthan parai Ar

Remarks: It grows to a fairly large size and is known to attain 12 kg in weight.

Relative abundance: Common.

Puntius chola (Hamilton)

Common name: Chola barb, swamp barb, green barb

Vernacular name: Kan. - Dhoddakarse; Mal. - Poovali kendai, koroon, Tam. - Korron, Putti kendai, Macha kendai, Vannathi

Diagnosis: Deep bodied; no bands; one pair of barbels; dorsal fin ossified, spine smooth; lateral line complete with 24 to 29 scales; black spot after gill opening and caudal base.

Earlier records: North-eastern parts of Bengal (Hamilton, 1822), Bombay (Valenciennes, 1842), Malabar (Day, 1878, 1889), Chitaldrug dist, Karnataka (Hora, 1936), Darna river, Deolali, Maharashtra (Hora and Misra, 1937), Deolali (Hora and Misra, 1938), Poona (Hora and Misra, 1942), Bhavani and Moyar rivers, Nilgiri Hills (Rajan, 1956), Bhadra river at Bhadravati (David, 1956), Moyar river, Nilgiri Hills (Rajan, 1963), Cardamon hills (Jayaram *et al*., 1976), Cauvery river, South India (Jayaram *et al*., 1982), Mavanhalla and Moyar (Manimekalan, 1998) throughout India.

Anamalai: Urilikal Ar

Remarks: Fairly common in coastal areas of Tamil Nadu and Kerala.

Relative abundance: Not common.

Puntius fasciatus fasciatus (Jerdon)

Common name: Melan barb

Diagnosis: Body elongate; 2 or 3 vertical colour bands on body; 4 barbels; dorsal ray weak and non osseous; lateral line complete with 18 - 20 scales; colour -body deep dull red with 3 drak vertical bands, the first below the whole of the dorsal fin base, 2nd commencing slightly behind the base of dorsal fin and the 3rd just before the base of caudal fin.

Earlier records: Streams of Malabar (Jerdon, 1849), Cochin (Day, 1865), Malabar (Day, 1865, 1878), Kottigehar village, Karnataka, Cauvery river, Coorg (Hora, 1937), Manimala river, Pampadampara, Kerala (Hora and Law, 1941), Coorg, Wynaad, Nilgiris (Hora, 1942), Puthuthottam estate, Valparai, Vannamudi bridge, Anaimalai hills, Ponnani drainage, Nelliampathi hills, Periyar drainage, Kerala (Silas, 1951).

Anamalai: Varakaliyar shoal, Koorampalli Ar, Panathiyar, Karuneer pallam, Urilikal Ar, Kallar, Ittli Ar, Itamalai Ar, Chinna Kallar, Vandal Ar,

Devi Ar, Kel Poonachi, Navamalai Ar, Periyathorai Ar (Kurumalai), Dattankanavai Ar, Sambathurai Ar, Kopparai kajam (Manjampatti), Sallimuthan parai Ar

Remarks: Commonly found in the region. It breeds easily in ponds and tanks.

Status: Common.

Puntius filamentosus (Valenciennes)

Common name: black spot barb

Vernacular name: Mal. - Kachi parval; Tam. - Chevalle, Machakendai

Diagnosis: Elongate body; 1 pair rudimentary maxillary barbels; dorsal ray prolonged upto caudal base; third ray longest; lateral line complete with about 21 scales; black spot on caudal peduncle.

Earlier records: Alleppey, Kerala (Valenciennes, 1844), rivers in Canara (Jerdon, 1849), rivers in Canote in Malabar (Jerdon, 1849), Malabar (Day, 1865, 1868, 1878, 1889), Madras (Raj, 1916), Bombay presidency (Spence and Prater, 1932), Coorg (Hora, 1937), Sharavaty river Jog falls (Hora, 1937), Manimala river, Peruntenaruvi, Kallada river, Kulathupuzha, Central Kerala (Hora and Law, 1941), Kallar stream, Trivandrum, Kerala (Hora and Nair, 1941), Coorg, Wynaad, Nilgiris (Hora, 1942), Coorg, Nilgiris, Wynaad (Hora, 1944), Nelliampathi Hills, Periyar drainage, Kerala (Silas, 1951), Bhavani and Moyar rivers, Nilgiri Hills (Rajan, 1956), Bhadra river at Bhadravati (David, 1956), Chapora river, Pemem; Valur river, Arvalem water falls, Goa (Tilak, 1973), Cardamom hills (Jayaram *et al*., 1976), Cauvery river, S. India (Jayaram *et al*., 1982), Sinkaara, Mudumalai (Manimekalan, 1998)

Anamalai: Karuneer pallam, Paraiyan kadau (Kumiti parai), Navamalai Ar

Remarks: It also breeds in ponds. 4 black vertical stripes on a pink body in juveniles. As the fish matures, the stripes disappear and only one remains as a horizontal oval spot on the caudal peduncle.

Relative abundance: Not common.

Puntius jerdoni (Day)

Common name: Jerdon's Crap

Vernacular name: Kandai meen

Diagnosis: Moderately deep bodied; no bands or spots; 2 pairs of barbels; dorsal ray weak and articulated; lateral line complete with 26-32 scales, predorsal scales 12.

Earlier records: Rivers of Canara, Mangalore, Karnataka (Day, 1870), Deccan (Day, 1876, 1878, 1878, 1989), Coorg, Wynaad, Nilgiris (Annandale, 1919), Tunga river at Shimoga (Hora, 1937), Poona (Hora and Misra, 1942), Coorg, Wynaad, Nilgiris (Hora, 1942), Karnataka (Hora, 1942), Anamalai (Silas, 1951), Krishna river at Wai, Maharashtra (Silas, 1953), Bhavani and Moyar rivers, Nilgiris (Rajan, 1955), Bhavani and Moyar rivers, Nilgiri Hills (Rajan, 1956), (David, 1956) Bhadra river at Bhadravati), Cauvery river, South India (Jayaram *et al*., 1982).

Range: Deccan, along the Western Ghats from Wai to Anaimalai hills (Maharashtra, Karnataka, Tamil Nadu and Kerala)

Anamalai: Sallimuthan parai Ar

Remarks: The presence of tubercles on the snout is due to sexual dimorphism. It forms a major fishery in Anjanapur reservoir (Shimoga district, Karnataka). It grows upto a length of 450 mm.

Status: The catch has declined in recent years, needs monitoring.

Puntius melanampyx (Day)

Common name: Tiger barb, black spot barb

Vernacular name: Mal. - Kachi parval; Tam. - Chevalle, Machakendai

Diagnosis: Elongate body; 1 pair rudimentary maxillary barbels; dorsal ray prolonged upto caudal base; third ray longest; lateral line complete with about 21 scales; black spot on caudal peduncle.

Earlier records: Karnataka (Day, 1870), Deccan (Day, 1876, 1878, 1878, 1989; Hora, 1942), Coorg, Wynaad, Nilgiris (Annandale, 1919), Tunga river at Shimoga (Hora, 1937), Nilgiris (Hora, 1942), Anamalai (Silas, 1951), Bhavani and Moyar rivers, Nilgiris (Rajan, 1955), Bhavani and Moyar rivers, Nilgiri Hills (Rajan, 1956), Cauvery river (Jayaram *et al*., 1982).

Range: Deccan, along the Western Ghats

Anamalai: Meenmettu pallam, Varakali Ar Camp site, Paramankudi, Kallar, Ittli Ar, Chinna Kallar, Pannikuli Ar, Kadamparai Ar, Navamalai Ar, Sambathurai Ar, Thoni Ar, Kopparai kajam (Manjampatti), Manal Allai (Manjampatti), Kumbur Ar, Azhanthoni, Sallimuthan parai Ar.

Tor khudree (Sykes)

Common name: Deccan Mahseer, Yellow Mahseer

Vernacular name: Kan. - Bili meen; Tam. - Biriga, Poomeen, Peruval

Diagnosis: Body elongate; snout covered with indistinct tubercles; eyes visible from underside of head; 4 barbels; lower lip protruding;

pectoral fins short; large scales; lateral line scales 25-27; colour - silvery with yellow below lateral line, belly bluish, eyes red, fins bluish grey.

Earlier records: Mula-Mutha river, Pune (Sykes, 1841) mountain streams of Malabar (Jerdon, 1849), South Canara down the Western Ghats to Travancore hills (Day, 1878, 1889), Deolali (Hora and Misra, 1937), Deolali (Hora and Misra, 1938), Mula-Mutha river (Poona Hora, 1942) Mysore (Hora, 1942) Ulhas, Cis ghat, north of Bombay (Hora, 1943), South Canara, Travancore hills (MacDonald, 1944), Manjira River Nizamsagar (Mahmood and Rahimullah, 1947), Moola-mootha river, Poona (Menon, 1949), Anamalai Hills (Silas, 1951), Bhavani and Moyar rivers and some of their tributaries (Rajan, 1955), Kolhapur, Maharashtra (Kalawar and Kelkar, 1955), Pykara and Moyar river, Nilgiris (Rajan, 1963), Krishna and Godavari rivers (David, 1963), Poona dist. Maharashtra (Tilak and Tiwari, 1976), Bhima, Krishna Koyna and Indrayani rivers of Maharashtra (Kulkarni, 1979), Cauvery river (Jayaram, 1982), Moyar near Kargudi, Mudumalai (Manimekalan, 1998).

Anamalai: Chinnar near Peryamula parai, Karuneer pallam, Meen parai, Paraiyan kadau (Kumiti parai), Urilikal Ar, Poovar, Kadamparai Ar

Remarks: It grows to 450mm and about 22 kg in weight.

Relative abundance: Threatened.

Salmostoma boopis (Day)

Common name: Velichi

Vernacular name: Boopis razorbelly minnow

Diagnosis: Body elongate and compressed; mouth oblique; lower jaw with a symphysial process; dorsal fin inserted well in advance to anal fin; scales large; lateral line curved slightly with 39-42 scales; colour-silvery.

Earlier records: South Canara (Day, 1873), Malabar (Day, 1878, 1968), Cardamom and Agastya hill ranges of the Western Ghats (Jayaram *et al*., 1976), Poona dist. Maharashtra (Babu Rao and Yazdani, 1977), Ujani wetland, Krishna river, Maharashtra (Yazdani and Singh, 1990) Western Ghats - Maharashtra and South Canara.

Anamalai: Navamalai Ar, Sallimuthan parai Ar

Remarks: It grows to 120 mm. length and is of no fishery importance.

Status: Common.

Barilius bakeri Day

Common name: Baril

Vernacular name: Paral

Diagnosis: Body deep; barbels absent; well developed tubercles on snout and lower jaw; dorsal fin inserted before the anal fin; lateral line scales 38; predorsal scales 16; colour - greyish with dorsal, pectoral and anal fins grey with white edges.

Earlier records: Mundakayam, Kerala (Day, 1865), Wynaad, Kerala and Malabar (Day, 1878, 1889), Cardamon Hills, South India (Jayaram *et al*., 1976), Western Ghats-Kerala.

Anamalai: Varakali Ar Camp site, Karuneer pallam

Remarks: It grows to about 150 mm. It is of no fishery interest.

Status: Restricted.

Barilius barna Hamilton

Common name: Indian hill stream trout

Vernacular name: Paral

Diagnosis: Body deep; no barbels; well developed tubercles on snout; dorsal fin inserted in advance of anal fin; lateral line scales 39-42; predorsal scales 15 or 16; anal fin with 10 or 11 branched rays; colour - dull greenish with 7 to 11 well developed vertical dark bars.

Earlier records: Yamuna and Brahmaputra rivers, extreme branches of Ganges (Hamilton, 1822), Cauvery (Bleeker, 1853; Guenther, 1868), Tunga river at Shimoga (Hora, 1937), Godavari river system (David, 1963), Thungabhadra reservoir (David, 1979) Upto Tunga river.

Anamalai: Karuneer pallam, Meen parai, Urilikal Ar, Periya Kallar, Kel Poonachi, Navamalai Ar, Sambathurai Ar, Panjalingam Ar (Bellow the Falls)

Remarks: Grows upto 70 mm length and is good for angling.

Status: Common.

Barilius canarensis (Jerdon)

Common name: Jerdon's baril

Vernacular name: Indian hillstream trout

Diagnosis: Body deep; jaws short; well developed tubercles on head; barbels absent; dorsal fin inserted before the anal fin; lateral line with 38 scales; predorsal scales 15; colour - back greenish, sides are golden, fins grey.

Earlier records: Canara (Jerdon, 1849; Day, 1878, 1889; Hora, 1942), Western Ghats - Karnataka and Kerala.

Anamalai: Varakaliyar shoal, Koorampalli Ar

Remarks: It grows to 150 mm. in length. It is of no fishery interest.

Status: Restricted.

Barilius gatensis (Valenciennes)

Common name: Hill trout, river carp

Vernacular name: Paral

Diagnosis: They are active stream fish with a deep body; 2 minute rostral barbels; dorsal fin inserted in advance of anal fin; tubercles on head well developed; lateral line with 40-41 scales; predorsal scales 15; colour-silvery grey with 13-15 vertical bars.

Earlier records: Peninsula of India (Valenciennes, 1844), Through India (Day, 1878, 1889), Bombay presidency (Spence and Prater, 1932), Travancore (Hora and Law, 1941), Poona, Bombay Presidency (Sutur, 1944), Anamalai Hills (Silas, 1951), Bhavani and Moyar rivers and some of their tributaries (Rajan, 1956), Silent Valley, Kerala (Rema Devi and Indra, 1986) Mavin halla, Mudumalai (Manimekalan, 1998).

Anamalai: Varakali Ar Camp site, Karuneer pallam, Paraiyan kadau (Kumiti parai), Kallar, Ittli Ar, Periya Kallar, Chinna Kallar, Vandal Ar, Devi Ar, Pannikuli Ar, Kel Poonachi, Navamalai Ar, Dattankanavai Ar, Chinnar (Kattal Parai Ammam paguthi), Manal medu, Kopparai kajam (Manjampatti), Manal Allai (Manjampatti), Azhanthoni, Sallimuthan parai Ar

Remarks: Grows to 150 mm.

Relative abundance: Rare.

Danio aequipinnatus (McClelland)

Common name: Blue or Giant Danio

Vernacular name: Tel.- Vannathipodi, Tam. - Selai parvai, Vannathi podi

Diagnosis: Body elongate and compressed; pre-orbital spine present on the lachrymal bone; mouth directed upwards; 4 short barbels; lateral line complete with 35-37 scales; predorsal scales 15; colour-brilliant blue with well developed lateral dark blue bands, on the sides of which are thinner golden bands, fins bright orange.

Earlier records: Assam (McClelland, 1839), throughtout India (Day, 1878, 1889), Deolali (Deolali) Deolali (Hora and Misra, 1938), Anamalai and Nelliampathi Hills (Silas, 1951), Bhavani and Moyar rivers and some of their tributaries (Rajan, 1956), Kolhapur dist. Maharashtra (Kalawar and Kelkar, 1956), Cardamom and Agastya hill ranges of the Western Ghats (Jayaram *et al*., 1976), Indrayani river, Poona dist. Maharashtra (Yazdani and Mahabal, 1976), Poona dist. Maharashtra (Babu Rao and Yazdani, 1977), Borivli, Bombay (Singh and Yazdani, 1988), Ujani wetland, Krishna river, Maharashtra (Yazdani and Singh, 1990), Dhule

dist. Maharashtra (Singh, 1990), Nasik dist. Maharashtra (Singh and Yazdani, 1991), Mudumalai (Manimekalan, 1998).

Anamalai: Through the sanctuary

Remarks: It is widely distributed throughout India and is an attractive aquarium fish. It grows to 150 mm. and prefers hill streams.

Relative abundance: Very common.

Parluciosoma daniconius (Hamilton)

Common name: Blackline rasbora

Vernacular name: Tam. - Jobidayee, Narangi

Diagnosis: Body oblong and compressed; lips simple; pectoral fins short; lateral line complete with 34 scales; colour - silvery with the back being dark, a distinct dark band on the sides flanked with gold.

Remarks: It grows to 100 mm. in length and is quite commonly found in almost all the aquatic habitats.

Earlier records: Rrivers of southern Bengal (Hamilton, 1822), Cuavery basin (Day, 1878, 1889), Yenna river, Satara dist. Bombay Presidency (Annandale, 1919), Deccan and neighbouring districts of Bombay Presidency (Spence and Prater, 1932), Chitaldurg dist. Mysore (Hora, 1936), Tunga river, Mysore State (Hora, 1937), Deolali (Hora and Misra, 1938), Anamalai Hills (Silas, 1951), (Brittan, 1954), Bhavani and Moyar rivers and some of their tributaries (Rajan, 1956), Kolhapur dist. Maharashtra (Kalawar and Kelkar, 1956), Cardamom and Agastya hill ranges of the Western Ghats (Jayaram *et al*., 1976), Indrayani river, Poona dist. Maharashtra (Yazdani and Mahabal, 1976), Poona dist. Maharashtra (Babu Rao and Yazdani, 1977), cauvery river system (Jayaram *et al*., 1982), Borivli, Bombay (Singh and Yazdani, 1988), Dhule dist. Maharashtra (Singh, 1990), Mudumalai (Manimekalan, 1998).

Anamalai: Through the sanctuary

Relative abundance: Very common.

Parluciosoma labiosa (Mukerji)

Common name: Slinder resbora

Vernacular name: Ovaree candee

Diagnosis: Body elongate and compressed; lower lip hypertrophied, projects beyond the jaw with 3 distinct lobe-like structures. Lateral line incomplete, extending upto end of anal fin; colour - pale brownish with a broad lateral band on the sides.

Earlier records: Deolali, Nasik (Mukerji, 1935), Poona (Fraser, 1937), Deolali (Hora and Misra, 1938), Peninsular India (Brittan, 1954).

Anamalai: Paraiyan kadau (Kumiti parai)

Remarks: Grows upto 80 mm. It has not been reported after the initial reports. Repeated surveys in the region by the author did not yield the fish.

Status: Endangered

Garra gotyla stenorhynchus (Jerdon)

Common name: Stone sucker, Nilgiri gara

Vernacular name: Mal.-Kallangkari; Tam.-Kal kaagan

Diagnosis: Body elongate and subcylindrical; depth of body 5 or more times in standard length; snout with a proboscis; 4 barbels; lateral line scales 32-35; predorsal scales 8-10; colour - greyish above and pale on the sides, a black spot on operculum.

Earlier records: Jerdon, 1849 (Bhavani river, Tamil Nadu), Cauvery river (Hora, 1921), Bhavani and Moyar rivers and some of their tributaries (Rajan, 1956), Nilgiri (Menon, 1964), Nasik dist. Maharashtra (Singh and Yazdani, 1991), Mudumalai (Manimekalan, 1998), Western Ghats - Krishna and the Cauvery river systems.

Anamalai: Chinnar (Kattal Parai Ammam paguthi), Manal medu, Manal Allai (Manjampatti), Kumbur Ar, Azhanthoni

Remarks: Grows to 150 mm. An ideal example for morphological adaptations in stream habitats.

Relative abundance: Common

Garra hughi Silas

Common name: Cardamon garra

Vernacular name: Mal.-Kallangkari; Tam.-Kal kaagan

Diagnosis: Body slender; depth of body 4.7 to 6.7 times in standard length; 4 barbels; lateral line with 36- 38 scales; back and belly without scales; colour - upper portion greyish, sides yellowish, a dark lateral band on the side, another one along the scaless dorsal part of body.

Earlier records: The distribution of *Garra hughi* Silas (1955) so far known from the Cardamom and Palani hills (Talwar and Jhingran, 1991; Jayaram, 1999) and this is the first record of this species to NBR.

Anamalai: Kallar, Poovar, Vandal Ar, Devi Ar, Kel Poonachi, Confluence of Vandi Ar and Vendaiyathar, Periyathorai Ar (Kurumalai), Dattankanavai Ar, Sambathurai Ar, Manal medu, Kopparai kajam

(Manjampatti), Manal Allai (Manjampatti), Kumbur Ar, Chinnar (Kattal Parai Ammam paguthi)

Range: Western Ghats - Cardamon and Palani hills.

Remarks: Grows to 70 mm. in length.

Status: Restricted.

Garra mcclellandi (Jerdon)

Common name: Stone carp

Vernacular name: Tam. - Kallukoravai

Diagnosis: Body subcylindrical; depth 4.3 to 5.7 times in standard length; mouth wide; snout with a transverse groove; 4 barbels; lateral line scales 38; predorsal scales 10; belly portion scales; colour – greyish with a black spot on operculum.

Earlier records: *Garra mcclellandi* was reported by many authors from Nilgiri hill ranges, Bhavani river and Manantoddy river at base of Neilgherry (Jerdon, 1849), Nierolay stream at base of Nilgiris (Rao, 1920), Wynaad (Silas, 1958), Nilgiri Biosphere Reserve (Daniels, 1993), Chooralmalai and Kanthampara of Chaliyar basin (Easa and Basha, 1995). The present collection from Mangalapatti and near Moyar village showed the range of extension to river Moyar.

Anamalai: Varakali Ar Camp site, Karuneer pallam, Meen parai, Paraiyan kadau (Kumiti parai), Urilikal Ar, Paramankudi, Itamalai Ar, Periya Kallar, Chinna Kallar, Kadamparai Ar, Navamalai Ar, Sambathurai Ar, Manal Allai (Manjampatti), Azhanthoni, Sallimuthan parai Ar

Remarks: Grows to a length of 170 mm. No fishery interest.

Status: Common.

Garra mullya (Sykes)

Common name: Stone carp, Mullya garra

Vernacular name: Tam-Kallu koravai

Diagnosis: Body depth 4 times in standard length; snout rounded and marked by a deep transverse groove; mouth small; 4 barbels; caudal fin emarginate; lateral line scales 32-34; predorsal scales 9-11; colour - upper portion dark, belly white, broad lateral band on the sides. A distinct red spot on operculum.

Earlier records: Bhima river, Daund, Pune district, Maharashtra (Sykes, 1841), Harang river, Coorg (Rao, 1920), Deolali (Hora and Misra, 1938), Anamalai Hills (Silas, 1951), Bhavani and Moyar rivers and some of their tributaries (Rajan, 1956), Kolhapur dist. Maharashtra (Kalawar and Kelkar, 1956), Cardamom and Agastya hill ranges of the

Western Ghats (Jayaram *et al*., 1976), Borivli, Bombay (Singh and Yazdani, 1988), Ujani wetland, Krishna river, Maharashtra (Yazdani and Singh, 1990), Dhule dist. Maharashtra (Singh, 1990), Moyar and Kakkanhalla, Mudumalai (Manimekalan, 1998).

Anamalai: Through the sanctuary

Remarks: Due to its wide distribution the specimens from different river systems show morphological variations in size of mental disc, shape, colouration, etc.

Relative abundance: Not common.

Balitora brucei Gray

Common name: Gray's stone loach

Diagnosis: Longer head; larger body scales; vent placed far in front of anal fin.

Earlier records: Mysore (Gray, 1830), Peninsular India (Hora, 1941), Mahabaleshwar and Wai, Satara district, Bombay state (Silas, 1953; Menon, 1987).

Anamalai: Manal medu

Remarks: Found in torrential streams of the Western Ghats.

Status: Not known.

Bhavania australis (Jerdon)

Common name: Western Ghat loach

Diagnosis: Head and anterior part of the body greatly depressed; gill openings restricted above base of pectoral fins; a deep groove between the upper lip and rostal fold.

Earlier records: Walliar, Nilgiris (Jerdon, 1849), Nilgiris, Wynaad and Bhavani (Day, 1877, 1889), Travoncore (Silas, 1951), Anamalai and Nelliampathi hill ranges (Western Ghats) (Silas, 1952). Extreme south of the Western Ghats, Karnataka, Nilgiris (Tamil Nadu) and Kerala.

Anamalai: Varakali Ar Camp site

Remarks: No commercial value.

Status: Not common.

Homaloptera montana Herre

Common name: Anamalai loach

Vernacular name: Pun - Saant-al

Diagnosis: Dorsal fin commencing behind origin of pelvic; pectoral reaching pelvic base; caudal slightly emarginate; 72 lateral line scales.

Earlier records: Puthutam Estate, Anamalai Hills (Herre, 1945; Hora, 1950), Peninsular India (Silas, 1951), Mahabaleshwar and Wai, Satara district, Bombay state (Silas, 1953), Silent valley, Kerala (Rema Devi and India, 1986) and Sayivala, New Amarambalam (Bharathapuzha basin) (Menon, 1987).

Anamalai: Itamalai Ar, Poovar

Remarks: Found in mountain streams.

Status: Endemic.

Travancoria elangata Pethiyagoda & Kottelat

Common name: Anamalai loach

Vernacular name: Pun - Saant-al

Diagnosis: *Travancoria elongata is* distinguished from its only congener, *T. jonesi* by havingamore slender body (body depth 8.2-10.5% SL,vs. 12.6-14.7%, a longer and more slender caudal peduncle (depth 4.7-6.3 times in its length, vs. 2.6-3.4), and in having the lobes of the rostral cap between the rostral barbels present, but not developed into barbel-like projections.

Anamalai: Urilikal Ar

Earlier records: Pethiyagoda and Kottelat (1994) described *Travancoria elangata* from Chalakudy river near Vettilappara. After Pethiyagoda and Kottelat (1994) this species has not been reported from anywhere. The present record showed its range of extension to Anamalai hills.

Remarks: Range of extention to the Anamalai hills.

Status: Threatened.

Travancoria jonesi Hora

Common name: Travancore loach

Diagnosis: Head and body depressed; tail compressed side to side;mouth small and inferior and semicircular.

Earlier records: Streams within a radius of 5 miles from Pampadampara, Peerumedu, Kerala (Hora, 1941; Silas, 1953; Menon, 1987)

Range: Western Ghats - Kerala high ranges and Anamalai hills

Anamalai: Kallar

Remarks: Found in mountain streams.

Status: Not common.

Nemacheilus denisoni denisoni Day

Common name: Loach

Vernacular name: Assari meen

Diagnosis: 8 branched dorsal fin rays; pelvic touching the anal opening; caudal deeply emarginate with rounded lobes; lateral line imcomplete, ending in front of dorsal fin; body with varying number of brown bands, more distinct behind dorsal fin; predorsal distance 22.5 - 55.5% of SL.

Earlier records: Bhawany river, base of Nilgiris (Day, 1867), Nilgiri (Gunther, 1868), Malabar and coorg (Day, 1872, 1873, 1878, 1889), Tunga river at Shimoga (Hora, 1937), Deolali (Hora and Misra, 1938), Moola-mootha river, Poona (Menon,1949), Mahabaleshwar, Satara dist. Bombay State (Silas, 1953), Bhavani and Moyar rivers and some of their tributaries (Rajan, 1956), Kolhapur dist. Maharashtra (Kalawar and Kelkar, 1956), Indrayani river, Poona dist. Maharashtra (Yazdani and Mahabal, 1976), Coorg dist., Karnataka (Raghunathan, 1989), Dhule dist. Maharashtra (Singh, 1990), Nasik dist. Maharashtra (Singh and Yazdani, 1991), Peninsular India, Bastar (Madhya Pradesh), Pamba and the Kollur drainages of Kerala and Karnataka states, Mudumalai (Manimekalan, 1998).

Anamalai: Dattankanavai Ar, Kopparai kajam (Manjampatti), Kumbur Ar, Sallimuthan parai Ar

Remarks: Grows upto a maximum length of 5 cm. commonly caught by tribals using traditional methods.

Relative abundance: Common.

Noemacheilus guentheri Day

Common name: Loach

Vernacular name: Assari meen

Diagnosis: 8 branched dorsal fin rays; lateral lien almost complete, reaching upto tip of the anal fin; caudal forked; body with 2 - 3 rows of large yellow spots edged with black.

Earlier records: Rivers along the lower slopes and base of the Neilgherry hills (Day, 1867), Wynaad (Day, 1872, 1878, 1889; Hora, 1941), Bhavani and Moyar rivers and some of their tributaries (Rajan, 1956), Silent valley, Kerala (Rema Devi and India, 1986), Nilgiri Biosphere Reserve (Daniels, 1993). Western Ghats-Kallar, Pamba, Periyar, Bharathapuzha rivers and Cauvery system in the Nilgiris and Mysore.

Anamalai: Varakali Ar Camp site, Karuneer pallam, Confluence of Vandi Ar and Vendaiyathar, Sambathurai Ar

Remarks: Length 56 mm. SL. It has no commercial importance.

Status: Common.

Noemacheilus monilis Hora

Common name: Loach

Vernacular name: Assari meen

Diagnosis: 7 branched dorsal rays; long thread- like barbels; caudal deeply forked; a distinct monoliform black band along line from tip of snout to base of caudal fin.

Earlier records: Bhavani river, 10 miles from Mettupalayam, base of Nilgiris (Hora, 1921), Bhavani and Moyar rivers and some of their tributaries (Rajan, 1956), Bhavani at Mukkali (Easa and Basha, 1995).

Anamalai: Chinnar (Kattal Parai Ammam paguthi), Sallimuthan parai Ar

Remarks: Maximum length 48 mm. No commercial fishery value.

Status: Restricted distribution.

Noemacheilus moreh (Sykes)

Common name: Striped loach

Vernacular name: Tam-Asarai meen

Diagnosis: 9 - 10 branched dorsal fin rays; lateral line incomplete, ending opposite to posterior end of dorsal fin.

Earlier records: Western Ghats (Sykes, 1841), Peninsular India (Day, 1970, 1872, 1878, 1889), (Annandale, 1919), Deolali (Hora and Misra, 1938), Poona, Bombay State (Hora, 1942), Moola- mootha river, Poona (Menon, 1949) Dhule dist. Maharashtra (Singh, 1990).

Anamalai: Urilikal Ar

Remarks: Maximum 44 mm. SL.

Status: Common in hill streams.

Noemacheilus ruppelli (Sykes)

Common name: Striped Loach

Vernacular name: Assari meen

Diagnosis: Large-sized body; 10 branched dorsal rays; long snout; prominent eyes; long barbels; 18 -19 brownish vertical bands, reaching below lateral line but not the ventral side.

Earlier records: Deccan (Sykes, 1841; Day, 1878, 1879), Mysore (Rao, 1920), Poona, Bombay State (Hora, 1942), Kolhapur dist. Maharashtra

(Kalawar and Kelkar, 1956), Indrayani river, Poona dist. Maharashtra (Yazdani and Mahabal, 1976).

Anamalai: Kopparai kajam (Manjampatti), Sallimuthan parai Ar

Remarks: Max length 74 mm SL.

Status: Not common, needs monitoring.

Lepidocephalus thermalis (Valenciennes)

Common name: Malabar loach

Vernacular name: Mal-Ayira; Tam-Assaree, Assari meen,

Diagnosis: Body elongate; mouth inferior; 6 barbels;dorsal fin inserted anterior to pelvic fin; caudal fin truncate; scales present on anterior of pectoral fin base; colour - greyish green with 8 to 10 irregular blotches on sides; dorsal and anal fins with rows of spots.

Earlier records: Malabar (Valenciennes, 1846; Day, 1878, 1889), Yenna river, Satara dist.Bombay Presidency (Annadale, 1919), Tunga river at Shimoga (Hora, 1937), Anamalai Hills (Silas, 1951), Kolhapur dist. Maharashtra (Kalawar and Kelkar, 1956), Cardamom and Agastya hill ranges of the Western Ghats (Jayaram *et al*., 1976), Indrayani river, Poona dist. Maharashtra (Yazdani and Mahabal, 1976), Silent Valley, Kerala (Rema Devi and Indira, 1986), Coorg dist., Karnataka (Raghunathan, 1989), Dhule dist. Maharashtra (Singh, 1990), Nasik dist. Maharashtra (Singh and Yazdani, 1991), Mudumalai (Manimekalan, 1998).

Anamalai: Varakaliyar shola, Koorampalli Ar, Meenmettu pallam, Varakali Ar Camp site, Vandal Ar, Devi Ar, Pannikuli Ar, Kel Poonachi, Sallimuthan parai Ar

Remarks: Grows upto 80 mm. This species exhibits a wide variation in colour pattern in different drainages.

Relative abundance: Not common

Mystus armatus (Day)

Common name: Catfish

Vernacular name: Keluthi

Diagnosis: Body elongate and compressed; head compressed; branchiostegal rays 10; 8 barbels, maxillary barbels reach base of pelvic fin; adipose fin short; colour -brownish on back, lighter on sides, with a dark blotch on caudal fin base.

Earlier records: Cochin (Day, 1865), Day (1877, 1889), throughout India (Misra, 1976), Cardamom and Agastya hills of the Western Ghats

(Jayaram *et al*., 1976), Coorg dist., Karnataka (Raghunathan, 1989) Wynaad hils.

Anamalai: Ambili cheak Dam, Kolikamuthi pallam Elephant camp

Remarks: Grows to about 140 mm. and is of minor fishery importance.

Status: Restricted.

Mystus cavasius (Hamilton)

Common name: Gangetic mystus, Dwarf catfish

Vernacular name: Tam. - Solai kelunthi

Diagnosis: Body elonagte and compressed; head conical; occipital process extends to dorsal fin base; branchiostegal rays 6; 8 barbels, maxillary barbels extend to beond caudal fin base; dorsal spine weak; colour - Greyish with a longitudinal stripe.

Earlier records: Gangetic provinces (Hamilton, 1822), Coorg and Malabar (Day, 1877, 1889), Deolali, Nasik dist. (Hora and Misra, 1938), Poona, Bombay State (Hora, 1942), Bhavani and Moyar rivers and some of their tributaries (Rajan, 1956), Kolhapur dist. Maharashtra (Kalawar and Kelkar, 1956), Cardamom and Agastya hills of the Western Ghats (Jayaram *et al.*, 1976), Indrayani river,Poona dist. Maharashtra (Yazdani and Mahabal, 1976), (Jayaram, 1977), Nasik dist. Maharashtra (Singh and Yazdani, 1991), Mudumalai (Manimekalan, 1998).

Anamalai: Kolikamuthi pallam Elephant camp, Kolikamuthi pallam, Aliyar Dam

Remarks: It grows to about 500 mm. and forms a major fishery in some areas.

Relative abundance: Not common.

Mystus montanus (Jerdon)

Common name: Wynaad mystus

Vernacular name: Tam.-Keluthi, Meesai meen

Diagnosis: Body elongate and compressed; head depressed; occipital process reaching dorsal fin base; dorsal spine weak and serrated on inner edge; 8 barbels, maxillary barbels extend to anal fin base; branchiostegal rays 10; colour - silvery on back with a dark spot on caudal fin base, a bluish spot on shoulder.

Earlier records: Jerdon (1849) described *Mystus montanus* from Manantovady, Wynaad, Kerala (Day, 1877, 1889). Later it was reported from Poona, Bombay Presidency (Suter, 1944), Cardamom and Agastya hills of the Western Ghats (Jayaram *et al*., 1976). Easa and Basha (1995)

reported from Mananthavady, Panamarampuzha of Kabini basin. According to Jayaram (1981, 1999) and Talwar and Jhingran (1991) this species had a distribution from Maharashtra, Karnataka and Kerala (Wynaad hills) of Western Ghats. In the present study also this species was collected from Hebballa and Sarathi hole, Kabini river basin in Karnataka part of NBR

Range: Western Ghats- Maharashtra, Karnataka and Kerala (Wynaad hills)

Anamalai: Varakali Ar Camp site

Remarks: It grows to a length of about 150 mm. and forms a minor fishery in the region.

Status: Common.

Ompok bimaculatus (Bloch)

Common name: Indian butter-catfish

Vernacular name: Silaivhalai, Chottavala

Diagnosis: Body elongate and strongly compressed; mouth large and oblique; 4 barbels, maxillary barbels extend to anal fin; anal fin long with 57-58 branched rays; colour - silvery with a large shoulder spot on the lateral line, a small blotch on the caudal peduncle.

Earlier records: Malabar (Bloch, 1797, Day, 1877, 1889), Deolali, Nasik dist (Hora and Misra, 1938), (Kolhapur dist. Maharashtra (Kalawar and Kelkar, 1956), Malabar, Kerala (Misra, 1976), Indrayani river, Poona dist. Maharashtra (Yazdani and Mahabal, 1976), Ujni wetland, krishna river, Maharashtra (Yazdani and Singh, 1990) Ombatta swamp (Manimekalan, 1998).

Anamalai: Paramankudi, Itamalai Ar, Aliyar Dam

Remarks: It grows to about 450 mm. It is found in rivers, tanks and ponds.

Relative abundance: Common in Ombatta swamp.

Ompok malabaricus (Valenciennes)

Common name: Goan catfisf

Diagnosis: Body elongate and compressed; 4 barbels, maxillary barbels long, extending slightly beyond pelvic fin origin; pectoral spine strong; anal fins with 63 - 69 branched rays; colour - greyish with a black spot on operculum.

Earlier records: Valenciennes (1839) described *Ompok malabaricus* Malabar. Later Haig (1951) reported this species from Goa and Trivandrum (Misra, 1976; Jayaram, 1981, 1999; Talwar and Jhingran,

1991) and Ajithkumar *et al*. (1999) reported this species from Chalakudy river, Kerala. Present record showed its presence in Nulpuzha.

Anamalai: Paramankudi, Aliyar Dam

Remarks: It grows to a length of about 500 mm.

Status: Restricted.

Glyptothorax housei Herre

Diagnosis: Body elongate and head depressed; lips papillated; 8 barbels, maxillary barbels extend slightly beyond pectoral fin base; dorsal fin inserted nearer snout tip; skin smooth; adhesive apparatus well developed, longer than broad and devoid of a central pit; colour - reddish with yellow mottlings.

Earlier records: Anamalai hills, Western Ghats (Herre, 1942, Silas, 1951, Misra, 1976)

Anamalai: Urilikal Ar, Kallar, Poovar

Remarks: 100 mm. in length.

Status: Rare.

Clarias dayi (Valenciennes)

Comman name: Malabar Clariid and Magur

Vernacular name: Tam-Masarai

Diagnosis: Body elongate, head depressed; mouth rather terminal; 8 barbels, short, not extending beyond eyes; nasal barbels shorter than half of head length; dorsal fin inserted behind pectoral fin tip; pectoral spine strong and serrated on its outer edge only; color dark on back, lighter on side.

Earlier records: After Hora (1936) described *Clarias dayi* from Wynaad, it has not been recorded from any parts of the India. Many workers have been working in Western Ghats of Wynaad, Nilgiri hills since 1921 (Hora 1921, 1937, 1938, 1942; Jayaram, 1981; Jayaram *et al*., 1982; Menon 1992; Rajan, 1956; Rema Devi and Indra, 1988; Silas, 1951a, 1952, 1953a, 1954; Easa, 1995; Easa and Shaji, 1995) did not report this species. Manimekalan (1998) and in the present study this species was collected from Mudumalai Wildlife Sanctuary which showed the occurrence of the species in this area. It is a rediscovery after 62 years at a new place. It showed its range of extension to Mudumalai Wildlife Sanctuary.

Anamalai: Varakali Ar Camp site

Status: This species is critically endangered

Clarias dussumieri Valenciennes

Common name: Magur, Air-breathing catfish

Vernacular name: Mal. - Muzhi, Tam. - Masarai, Kan. - Hali meenu

Diagnosis: Body elongate, head depressed; mouth terminal; 8 barbels, maxillary barbels extend beyond pectoral fin base, nasal barbels extend to occipital fontanelle; dorsal fin inserted opposite pectoral fin tip; pectoral spine strong and serrated on outer edge only; colour - dark on back, lighter on sides.

Earlier records: Clarias dussumieri was recorded from Pondicherry (Valenciennes, 1840), Malabar (Day, 1877, 1889), Travancore (Hora, 1941), throught India (Misra, 1976), Chaliyar river basin and Kabini basin (Nulpuzha). Easa and Basha (1995) reported this species from Karimpuzha stream, Ombatta swamp Mudumalai (Maniekalan and Arunachalam, 2002).

Range: Ombatta swamp; Peninsular India: Karnataka and Kerala.

Anamalai: Varakali Ar Camp site

Remarks: It grows to about 230 mm.

Relative abundance: Rare.

Status: This species is critically endangered

Salmo gairdnerii gairdnerii Richardson

Common name: Rainbow trout

Diagnosis: Body elongate with both profiles arched; mouth large; dorsal fin inserted in advance of pelvic fin; adipose fin above anal fin; lateral line scales 127- 160; colour- steel blue with a brilliant reddish band along side, no spots below the lateral line.

Earlier records: Columbia river, Port Vancouver (Richardson, 1836), (Tilak and Sharma, 1982), Nilgiri (Menon *et al*., 1954).

Anamalai: Konal Ar

Remarks: It was introduced in select Indian waters in 1869, namely, Tamil Nadu (Nilgiris), Kashmir, Himachal Pradesh and Uttar Pradesh (Western Himalayas). It grows to about 380mm and is an excellent game fish.

Status: Restricted.

Salmo turtta fario Linnaeus

Common name: Brown trout

Diagnosis: Body strong with both profiles arched; mouth large; dorsal fin inserted in advance of pelvic fin; adipose fin above anal fin;

scalesminute embeded in skin; lateral line scales 115-130; colour-generally deep brown with green or blue hues.

Earlier records: Rivers of Switzerlands

Anamalai: Konal Ar

Remarks: It was introduced in Indian waters in 1869, Tamil Nadu (Nilgiris)

Status: Restricted.

Xenentodon cancila (Hamilton)

Common name: Freshwater Garfish

Vernacular name: Tam- Vellaimural, Kokku meen, Kolachi, Peechu kola

Diagnosis: Body very elongate and cylindrical; upper and lower jaws extended into a long beak; dorsal fin inserted before anal fin, with 15 - 18 rays; caudal fin truncate; colour - upper portion greenish, sides silvery, dorsal and anal fins dark edged.

Earlier records: Ponds and smaller rivers of Gangetic provinces (Hamilton, 1822) Mota Mola river, Poona (Sykes, 1841), Southern India (Day, 1865), Malabar (Day, 1877, 1889), Travancore and Cochin (Jenkins, 1909), Tunga river at Shimoga (Hora, 1937), Poona, Bombay State (Hora, 1942), Moola- mootha river, Poona (Menon, 1949), Kolhapur dist. Maharashtra (Kalawar and Kelkar, 1956), Indrayani river,Poona dist. Maharashtra (Yazdani and Mahabal, 1976), Cardamom and Agastya hills of the Western Ghats (Jayaram *et al*., 1976), Poona dist.Maharashtra (Tilak and Tiwari, 1976), Ujni wetland, krishna river, Maharashtra (Yazdani and Singh, 1990).

Anamalai: Navamalai Ar

Remarks: It is commonly found in rivers and streams. It grows to about 400 mm. in length.

Status: Common.

Chanda nama Hamilton

Common name: Glass perchlet

Diagnosis: Body strongly compressed; mouth large with the lower lip extended; scales minute; lateral line with over 100 scales; colour - transparent, eyes black, fins bright orange.

Earlier records: Ponds throughout Bengal (Hamilton, 1822), Malabar (Day, 1875, 1889), Deolali, Nasik dist. (Hora and Misra, 1938), Kolhapur dist. Maharashtra (Kalawar and Kelkar, 1956), Poona dist. Maharashtra

(Tilak and Tiwari, 1976), Dhulia dist. Maharashtra (Singh, 1990), Nasik dist. Maharashtra (Singh and Yazdani, 1991).

Anamalai: Karian shola cheak Dam, Chinnar near Peryamula parai

Remarks: It grows to a length of about 100mm and forms a major food item for the underpriviledged.

Status: Common.

Chanda ranga Hamilton

Common name: Glass fish

Diagnosis: Body strongly compressed; mouth oblique; preopercle smooth; lateral line with 47 - 63 scales; colour - transparent with a black shoulder spot.

Earlier records: *Pseudambassis ranga* was distributed throughout India (Day, 1889, 1989; Jayaram, 1981, 1999; Talwar and Jhingran, 1991). This species was reported from Ganges (Hamilton, 1822), Tunga river at Shimoga (Hora, 1937), Deolali, Nasik dist. (Hora and Misra, 1938), Poona, Bombay state (Hora, 1942), Moola-mootha river, Poona (Menon, 1949), Kolhapur dist. Maharashtra (Kalawar and Kelkar, 1956), Poona dist. Maharashtra (Tilak and Tiwari, 1976; Babu Rao and Yazdani, 1977), Kabini river, Kerala (Easa and Basha, 1995), Chalikal of Chaliyar river basin, Nulpuzha, and in Kabini river basin (Manimekalan, 2000).

Anamalai: Varakali Ar Camp site

Remarks: It is an excellent aquarium fish.

Status: Common

Pristolepis marginata Jerdon

Common name: Malabar Catoper

Vernacular name: Chutichi

Diagnosis: Body oblong and compressed; mouth terminal; opercle with 2 spines, preopercle and preorbital serrated; dorsal fin with 3 stout spines; lateral line interrupted on 21st scale; colour - greenish with black crossbands, pectoral fin yellowish.

Earlier records: Manantoddy river, north Malabar (Jerdon, 1848) hill ranges of Travancore (Gunther, 1864), Malabar (Day, 1889), Cardamom and Agastya hills of the Western Ghats (Malabaricus, 1976).

Anamalai: Karian shola cheak Dam, Chinnar near Peryamula parai, Meenmettu pallam, Varakali Ar Camp site, Varakaliyar Dam site, Meen parai, Paraiyan kadau (Kumiti parai), Aliyar Dam

Remarks: It grows to a length of about 140 mm. It is found in hill streams flowing from the ghats.

Status: Not common.

Oreochromis mossambica (Peters)

Common name: Tilapia

Vernacular name: Thilappi, Jilabi meen

Diagnosis: Body elongate and compressed; mouth large and terminal; dorsal fin inserted above base of pectoral fin with 15-16 spines; scales cycloid; scales in lateral series 30-32; colour - greyish with 3-4 dark blotches on side; dorsal fin with red margin, pectoral fin translucent.

Earlier records: Mozambique (Peters, 1852; Jones and Sarojini, 1952), Cardamom and Agastya hills of the Western Ghats (Jayaram *et al*., 1976), Borivli, Bombay (Singh and Yazdani, 1988), Ujni wetland, Krishna river, Maharashtra (Yazdani and Singh, 1990), India (introduced).

Anamalai: Karian shola cheak Dam, Erumaparai stream, Ambili cheak Dam, Kolikamuthi pallam Elephant camp, Kolikamuthi pallam, Varakaliyar shola, Koorampalli Ar, Chinnar near Peryamula parai, Meenmettu pallam, Varakali Ar Camp site, Varakaliyar Dam site, Paraiyan kadau (Kumiti parai), Urilikal Ar, Dattankanavai Ar, Sallimuthan parai Ar)

Remarks: It was introduced into the country in 1952 and and now spread at an alarming rate in almost all the major river systems. This is a matter of concern, especially in the Ujni reservoir (Krishna river basin), Maharashtra, where the fish catches show a predominence of this fish. This was not the case 5 years ago.

Status: Common.

Channa orientalis Bloch and Schneider

Common name: Brown snakehead.

Vernacular name: Para koravai, Manian koravai

Diagnosis: Body elongate; mouth large; pectoral fins extend to anal fin; dorsal fin rays 32-37; length of pelvic fin less than 50% of pectoral fin length; scales on longitudinal series 40 to 50; colour - dorsal side greenish, ventral side pale with bluish tinge. Pectoral fins with a series of vertical bands.

Earlier records: Bloch and Schneider (1801) described (type locality not known) this species and later report from Bengal (Hamilton, 1822) Malabar (Day, 1876, 1889), Travancore and Cochin (Jenkins, 1909), Deolali, Nasik dist. (Hora and Misra, 1938), Poona, Bombay State (Hora 1942), Poona dist. Maharashtra (Babu Rao and Yazdani, 1977), Silent Valley, Kerala (Rema Devi and Indra, 1986), Dhulia dist. Maharashtra

(Singh, 1990), Nasik dist. Maharashtra (Singh and Yazdani, 1991) Mudumalai (Manimekalan, 1998).

Anamalai: Sallimuthan parai Ar

Remarks: Fairly common species in the Western Ghats. It is of some fishery value in Maharashtra.

Relative abundance: Not common.

Glossogobius giuris (Hamilton)

Common name: Tank gobi

Vernacular name: Tam-Vuluvai

Diagnosis: Body elongate, anteriorly cylindrical and compressed; head depressed; mouth oblique; tongue bilobed; gill openings continued far forward; colour - yellowish brown without longitudinal lines, iris without process in eye.

Locality and range: Ombatta swamp; India.

Earlier records: This species desceribed from Gangetic provinces by Hamilton (1822) and later this were reported from Malabar (Day, 1876, 1889), Travancore and Cochin (Hora, 1937), Tunga river at Shimoga (Misra, 1942), Deolali, Nasik dist. Poona, Bombay State (Hora, 1942), Moola-mootha river, Poona (Menon, 1949), Bhavani and Moyar rivers and some of their tributaries (Rajan, 1956), Kolhapur dist. Maharashtra (Kalawar and Kelkar, 1956), Borivli, Bombay, (Singh and Yazdani, 1988), Coorg dist., Karnataka (Raghunathan, 1989), Indrayani river, Poona dist. Maharashtra (Yazdani and Mahabal, 1976), Poona dist. Maharashtra, (Babu Rao and Yazdani 1977), Dhulia dist. Maharashtra (Singh, 1990), Ujni wetland, Krishna river, Maharashtra (Yazdani and Singh, 1990), Nasik dist. Maharashtra (Singh and Yazdani, 1991).

Anamalai: Navamalai Ar

Remarks: It grows to about 250 mm. and is fairly common in the rivers of the Western Ghats. It prefers deeper waters.

Relative abundance: Rare.

Mastacembelus armatus (Lacepede)

Common name: Tyre-track spiny eel

Vernacular name: Mal-Mookkan arakan, Aaron; Tam-Kul aral,

Diagnosis: Body eel-like and slender; dorsal fin with 32-40 spines and 64-92 soft rays; dorsal and anal fins broadly joined to caudal fin; colour - brownish with wavy lines forming a network, dorsal and anal fins banded.

Distirbution: Lacepede described this species in 1800 (type locality not known), later this species reported from Malabar (Day, 1876, 1889), Tunga river at Shimoga (Hora, 1937), Deolali, Nasik dist. (Hora and Misra, 1938), Moola-mootha river, Poona (Menon, 1949), Anamalai Hills (Silas,1951), Bhavani and Moyar rivers and some of their tributaries (Rajan, 1956), Kolhapur dist. Maharashtra (Kalawar and Kelkar, 1956), Poona dist.Maharashtra (Tilak and Tiwari, 1976), Indrayani river,Poona dist. Maharashtra (Yazdani and Mahabal, 1976), Ujni wetland, Krishna river, Maharashtra (Yazdani and Singh, 1990), Moyar (Manimekalan 1998).

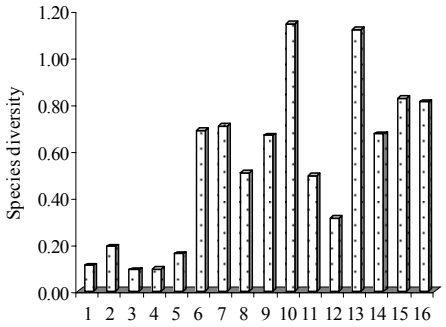
Anamalai: Paramankudi, Navamalai Ar

Remarks: It grows to 500 mm. and is one of the largest spiny eels in India.

Relative abundance: Rare.

14.6.2 Parambikulam river system

A total of 38 species were recorded from sixteen sites during the present study in Periyar river and its tributaries such as of Varakaliyar, Karuneer pallam and Parayankadau (Table 14.3). Maximum species richness (19) was recorded with high diversity value in Varakali Ar Camp site (1.15) and Karuneer pallam (1.12) and low species richness was recorded at Erumaparai stream (2), Karian shola cheak Dam (3) with low diversity values between 0.11, maximum abundance was noted at Ambili cheak Dam (252) followed by Kolikamuthi pallam (193), Kolikamuthi pallam Elephant camp (186) (Table 14.3; Figure 14.8).



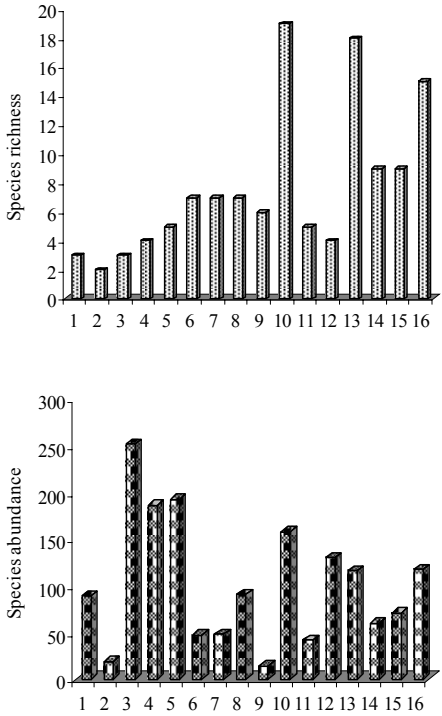


Figure 14.8: Fish diversity, richness and abundance in Paranbikulam river system.

Table 14.3: Distribution and relative abundance of fishes in Parambikulam river basin

S. No.	Species	Sites															
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	*Hypselobarbus dubius* Day													1.7	3.3		
2	*Hypselobarbus kolus* (Sykes)								3.3		7.0	57.1		11.2	20.0	38.0	10.2
3	*Labeo calbasu*									6.7							
4	*Neolissochilus anamalaiensissp.* nov.								3.3		2.5						1.7
5	*Neolissochilus wynaadensis* (Day)													2.6			
6	*Puntius carnaticus* (Jerdon)											2.4		1.7	1.7		
7	*Puntius chola* (Hamilton)																0.8
8	*Puntius fasciatus fasciatus* (Jerdon)						17.0	25.0					3.8	2.6			0.8
9	*Puntius filamentosus* (Valenciennes)													8.6		4.2	
10	*Puntius melanampyx*									40.0	1.3						
11	*Tor khudree* (Sykes)								3.3					3.4	1.7	7.0	1.7
12	*Tor khudree malabaricus*													4.3			2.5
13	*Tor putitora*											0.0		1.7			2.5
14	*Tor tor*										0.6	7.1					
15	*Tor anamalaiensis* sp. nov.													12.1			5.1
16	*Barilius bakeri* Day										0.6			10.3			
17	*Barilius barna* Hamilton													2.6	3.3		4.2

18	*Barilius canarensis* (Jerdon)						6.4	6.3							0.0		
19	*Barilius gatensis* (Valenciennes)										3.2			1.7	0.0	8.5	
20	*Danio aequipinnatus* (McClelland)					2.6	6.4	4.2		20.0	19.0		78.5	18.1	3.3	12.7	7.6
21	*Parluciosoma daniconius* (Hamilton)					1.0	14.9	14.6			9.5		13.1	2.6			
22	*Parluciosoma labiosa* (Mukerji)															8.5	
23	*Garra mcclellandi* (Jerdon)										1.3			1.7	20.0	4.2	7.6
24	*Garra mullya* (Sykes)		15.8	4.4	2.7	3.6	14.9	16.7	7.7	6.7	17.1		4.6	9.5	45.0		
25	*Bhavania australis* (Jerdon)										0.6						
26	*Travancoria elongata* Pethiyagoda & Kottelat																50.0
27	*Noemacheilus guentheri* Day										0.6			3.4			
28	*Noemacheilus moreh* (Sykes)																1.7
29	*Lepidocephalus thermalis* (Valenciennes)						4.3	6.3		6.7	9.5						
30	*Mystus armatus* (Day)			0.4	0.5												
31	*Mystus cavasius* (Hamilton)				1.1	0.5											
32	*Mystus montanus* (Jerdon)										7.6						
33	*Glyptothorax housei* Herre																2.5
34	*Clarias dayi*										0.6						
35	*Clarias dussumieri* Valenciennes										1.3						
36	*Chanda nama* Hamilton	2.2							4.4								
37	*Chanda ranga* Hamilton										3.2						
38	*Pristolepis marginata* Jerdon	3.4							9.9	6.7	1.3	7.1			1.7	12.7	

39	*Oreochromis mossambica* (Peters)	94.4	84.2	95.2	95.7	92.2	36.2	27.1	68.1	13.3	13.3	26.2				4.2	0.8

Among the 38 species, Hypselobarbus dubius, Puntius chola, Tor khudree malabaricus, Barilius bakeri, Parluciosoma labiosa, Bhavania australis, Mystus armatus, Mystus montanus, Glyptothorax housei, Clarias dayi, Clarias dussumieri recorded only in Parambikulam river system. An undescribed species from the genus Neolissochilus spp. was described from Paraiyankadau near Meenparai.

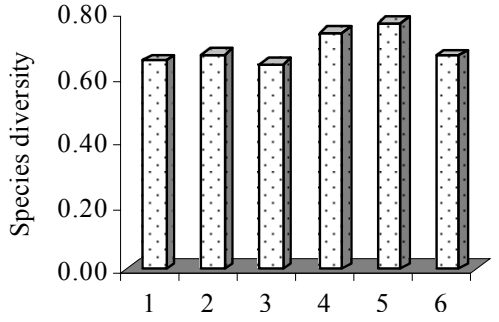
14.6.3 Idamalai Ar Basin

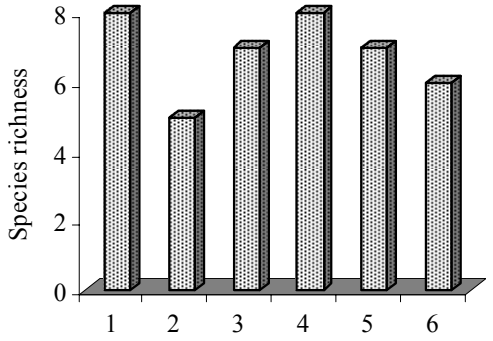
Twenty two species were recorded from 7 sites in the Idamalai Ar river system. Maximum species richness was recorded in Paramankudi (10) followed by Kallar (8) and Kallar (19) (Table 14.5). The maximum density was recorded in Kallar (190) followed by Paramankudi (77). Highest species diversity value was noted in Poovar (0.77), followed by Paramankudi (0.75) and low species diversity was recorded in Kallar (0.65) (Table 14.4; Figure 14.9). Fishes were collected from seven sites in Idamalai Ar river basin. Species like *Puntius cauveriensis*, *Homaloptera montana* are recorded only in Idamalai Ar system. *Puntius poovarensis* sp. nov., *Garra itamalaiyarensis* sp. nov. new two species collected from this river basin.

Table 14.4: Distribution and relative abundance of fishes in Idamalai Ar river basin

S. No.	Species	Study sites						
		17	18	19	20	21	22	23
1	*Hypselobarbus kolus* (Sykes)					32.8		
2	*Puntius carnaticus* (Jerdon)					1.5		
3	*Puntius cauveriensis* (Hora)						5.3	
4	*Puntius fasciatus fasciatus* (Jerdon)		10.0	23.9	5.4			31.4
5	*Puntius melanampyx*	1.3	8.4	9.9				13.7
6	*Puntius poovarensis* sp. nov.		12.1					
7	*Tor khudree* (Sykes)						7.9	
8	*Tor putitora*	1.3						
9	*Tor anamalaiensis* sp. nov.	19.5			16.2	19.4		
10	*Barilius barna* Hamilton					4.5		
11	*Barilius gatensis* (Valenciennes)		0.5	16.9		6.0		2.0
12	*Danio aequipinnatus* (McClelland)	5.2	3.7	16.9	5.4	1.5	34.2	15.7
13	*Garra hughi* Silas		54.2				13.2	
14	*Garra mcclellandi* (Jerdon)	9.1			48.6	10.4		31.4
15	*Garra mullya* (Sykes)	42.9		32.4	18.9	23.9	7.9	5.9

16	*Garra itamalaiyarensis* sp. nov.	2.6						
17	*Homaloptera montana* Herre				2.7		13.2	
18	*Travancoria jonesi* Hora		6.3					
19	*Ompok bimaculatus* (Bloch)	7.8			2.7			
20	*Ompok malabaricus* (Valenciennes)	9.1						
21	*Glyptothorax housei* Herre		4.7				18.4	
22	*Mastacembelus armatus* (Lacepede)	1.3			0.0			





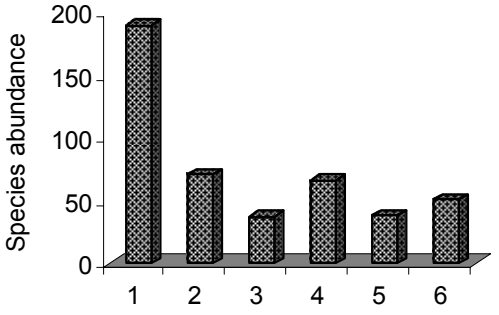


Figure 14.9: Fish diversity, richness and abundance in Idamalai Ar river basin.

14.6.4 Aliyar River Basin

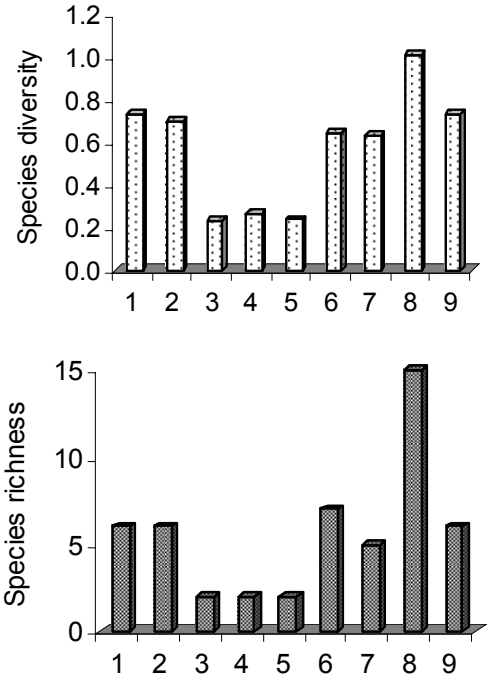
10 sites were sampled and 30 species were recorded with the diversity value of 1.35 in Aliyar river system. The maximum species richness (15) and diversity (1.01) were recorded at Navamalai Ar followed by Kel Poonachi (7, 0.65). Lowest species richness (2) recorded at Pambar (0.23), Konal Ar (0.24). The maximum species abundance was recorded at Navamalai Ar (123) followed by Kel Poonachi (90) and lowest species abundance was recorded at Pambar (9) (Table 14.5; Figure 14.10). The following species *Notopterus notopterus* (Pallas), *Cyprinus carpio communis* Linnaeus, *Salmo gairdenerii gairdnerii* (Richardson), *Salmo trutta fario* are showed restricted distribution to Aliyar river system in the IGWLS. *Barilius kadamparaiensis* sp. nov., a new species was collected from Vandal Ar and Pannikuli Ar.

Table 14.5: Distribution and relative abundance of fishes in Aliyar river basin

S. No.	Species	Study sites									
		24	25	26	27	28	29	30	31	32	33
1	*Notopterus notopterus* (Pallas)										23.08
2	*Anguilla bengalensis*									0.79	
3	*Catla catla*									1.57	
4	*Cirrhinus mirgala*									0.79	
5	*Cyprinus carpio communis* Linnaeus			14.29	77.78				1.92		
6	*Labeo rohit*										
7	*Neolissochilus wynaadensis* (Day)									0.79	
8	*Puntius carnaticus* (Jerdon)				22.22					2.36	7.69
9	*Puntius fasciatus fasciatus* (Jerdon)	8.89	13.79					35.56		4.72	
10	*Puntius*									7.87	

	filamentosus (Valenciennes)										
11	*Puntius melanampyx*			28.57					3.85	12.60	
12	*Tor khudree* (Sykes)								13.46		
13	*Salmostoma boopis* (Day)									1.57	
14	*Barilius barna* Hamilton							7.78		0.79	
15	*Barilius gatensis* (Valenciennes)	26.67	6.90	4.76				4.44		6.30	
16	*Babilius kadamparaiensi* ssp. nov.	15.56		23.81							
17	*Danio aequipinnatus* (McClelland)	13.33	20.69	4.76				38.89	25.00	15.75	
18	*Parluciosoma daniconius* (Hamilton)									16.54	
19	*Garra hughi* Silas	17.78	12.07					3.33			
20	*Garra mcclellandi* (Jerdon)								23.08	6.30	
21	*Garra mullya* (Sykes)		31.03					4.44	32.69	14.96	
22	*Lepidocephalus thermalis* (Valenciennes)	17.78	15.52	23.81				5.56			
23	*Mystus cavasius* (Hamilton)										7.69
24	*Ompok bimaculatus* (Bloch)										23.08
25	*Ompok malabaricus* (Valenciennes)										23.08
26	*Salmo gairdenerii gairdnerii* (Richardson)					70	95				
26	*Salmo trutta*					30	5				

	fario										
27	*Xenentodon cancila* (Hamilton)									0.79	
28	*Pristolepis marginata* Jerdon										15.38
29	*Glossogobius giuris* (Hamilton)									3.15	
30	*Mastacembelus armatus* (Lacepede)									2.36	



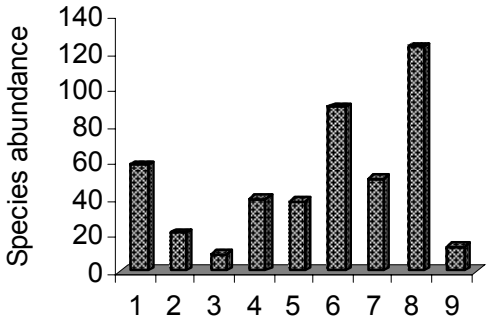


Figure 14.10: Fish diversity, richness and abundance in Aliyar river Basin.

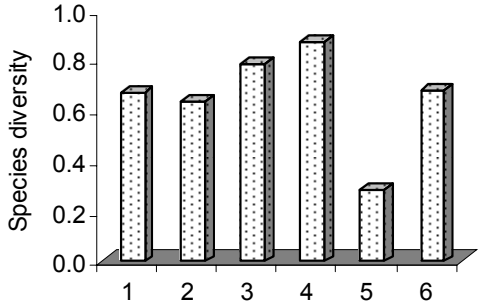
14.6.5 Thirumurthi River Basin

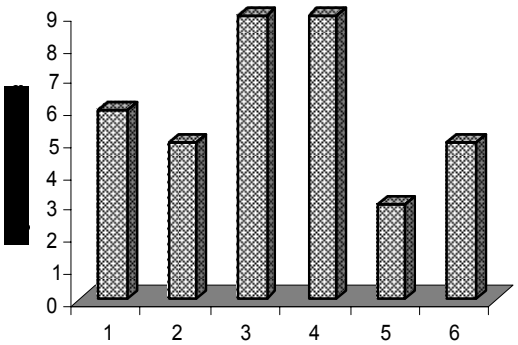
In the Indira Gandhi Thirumurthi river basin harbors the lowest species richness and diversity. Sixteen species were recorded from seven sites in Thirumurthi river river system (Table 14.6) with 1.12 diversity values. Maximum species richness (9) was recorded at Dattankanavai Ar (0.79), Smbathurai Ar (0.87) Low species richness was recorded in Thoni Ar (3) with low diversity value (0.28). Maximum abundance was observed in Periyathorai Ar (Kurumalai) (79) and the lowest abundance was recorded at Thoni Ar (26) (Table 14.6; Figure 14.11). *Puntius amphibius* (Valencienes), *Rasbora kannachiyaresis* sp. nov. were restricted to Thirumurthi river basin. An undescribed *Rasbora* species was collected from this river system.

Table 14.6: Distribution and relative abundance of fishes in Thirumurthi river basin

S. No.	Species	Study sites					
		34	35	36	37	38	39
1	*Hypselobarbus kolus* (Sykes)			3.57			
2	*Puntius amphibius* (Valencienes)	2.78					
3	*Puntius carnaticus* (Jerdon)			1.79			25.81
4	*Puntius fasciatus fasciatus* (Jerdon)		26.58	16.07	14.63		
5	*Puntius melanampyx*				4.88	19.23	
6	*Barilius barna* Hamilton				2.44		25.81
7	*Barilius gatensis* (Valenciennes)			3.57			
8	*Danio aequipinnatus* (McClelland)	38.89	2.53		17.07	46.15	12.90
9	*Parluciosoma daniconius* (Hamilton)		21.52	14.29	9.76	34.62	12.90

10	*Rasbora kannachiyaresis* sp. nov.	19.44					
11	*Garra hughi* Silas	5.56	27.85	32.14	24.39		
12	*Garra mcclellandi* (Jerdon)				7.32		
13	*Garra mullya* (Sykes)	13.89	21.52	3.57	14.63		22.58
14	*Noemacheilus denisoni denisoni* Day			3.57			
15	*Noemacheilus guentheri* Day	19.44			4.88		
16	*Oreochromis mossambica* (Peters)			21.43			





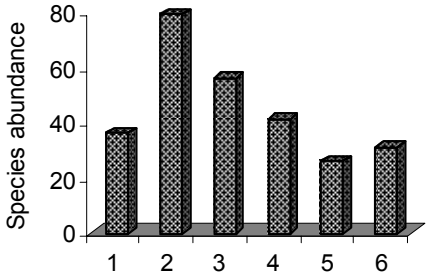


Figure 14.11: Fish diversity, richness and abundance in Thirumurthi river Basin.

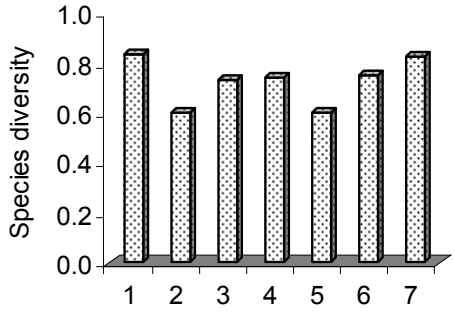
14.6.6 Amaravathi River Basin

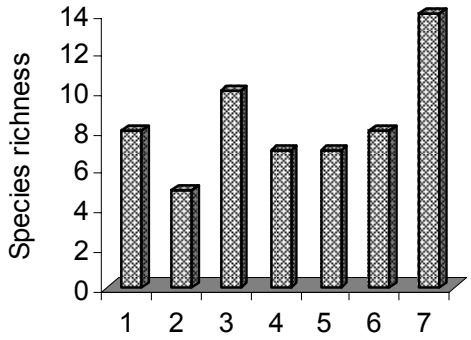
In total 16 species were recorded from 7 sites (Table 14.11). Maximum species richness (10) was recorded from Kopparai kajam (Manjampatti) and low species was recorded in Manal medu (5). The highest diversity value (0.84) was recorded at Chinnar (Kattal Parai Ammam paguthi). Maximum abundance 84 was recorded at Kopparai kajam (Manjampatti) and Sallimuthan parai Ar and the minimum abundance (23) was recorded at Manal Allai (Manjampatti) (Table 14.7; Figure 14.12). In the present study, twenty species were collected from seven sites. *Garra gotyla stenorhynchus*, *Balitora brucei, Noemacheilus monilis*, *Channa orientalis* found only in Amaravathi river system.

Table 14.7: Distribution and relative abundance of fishes in Amaravathi river basin

S. No.	Species	Study sites						
		40	41	42	43	44	45	46
1	*Neolissochilus wynaadensis* (Day)	22.22		5.81	4.35	4.26	26.92	1.16
2	*Puntius carnaticus* (Jerdon)							2.33
3	*Puntius fasciatus fasciatus* (Jerdon)			3.49				1.16
4	*Puntius melanampyx*			3.49	4.35	6.38	3.85	2.33
5	*Salmostoma boopis* (Day)							18.60
6	*Barilius gatensis* (Valenciennes)	11.11	17.65	6.98	17.39		3.85	31.40
7	*Danio aequipinnatus* (McClelland)	22.22		37.21	30.43	57.45	3.85	18.60
8	*Parluciosoma daniconius* (Hamilton)	7.41					3.85	
9	*Garra gotyla stenorhynchus* (Jerdon)	7.41	31.37		8.70	4.26	7.69	
10	*Garra hughi* Silas	7.41	35.29	30.23	26.09	14.89		
11	*Garra mcclellandi* (Jerdon)				8.70		19.23	2.33
12	*Garra mullya* (Sykes)	18.52	13.73	9.30		6.38	30.77	16.28
13	*Balitora brucei*		1.96					
14	*Noemacheilus denisoni denisoni* Day			1.16		6.38		1.16
15	*Noemacheilus monilis*	3.70						1.16

	Hora							
16	*Noemacheilus moreh* (Sykes)			1.16				
17	*Noemacheilus ruppelli* (Sykes)			1.16				
18	*Lepidocephalus thermalis* (Valenciennes)							1.16
19	*Oreochromis mossambica* (Peters)							1.16
20	*Channa orientalis* Bloch & Schneider							1.16





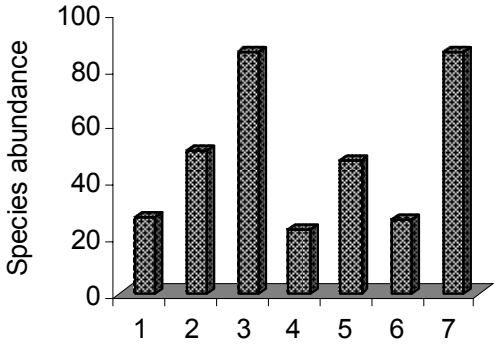


Figure 14.12: Fish diversity, richness and abundance in Amaravathi river Basin.

14.6.7 Species Similarity

Jaccard species similarity coefficient index showed, the species similarity index values were very low (> 0.3 were considered for species similarity). Similarly species composition sites for similar sites were presented in Table 14.16. Species similarity between the sites was very less among 46 sites of five river basins. Cluster analysis showed that similar species composition between the sites was in nearby position (Figure 14.13).

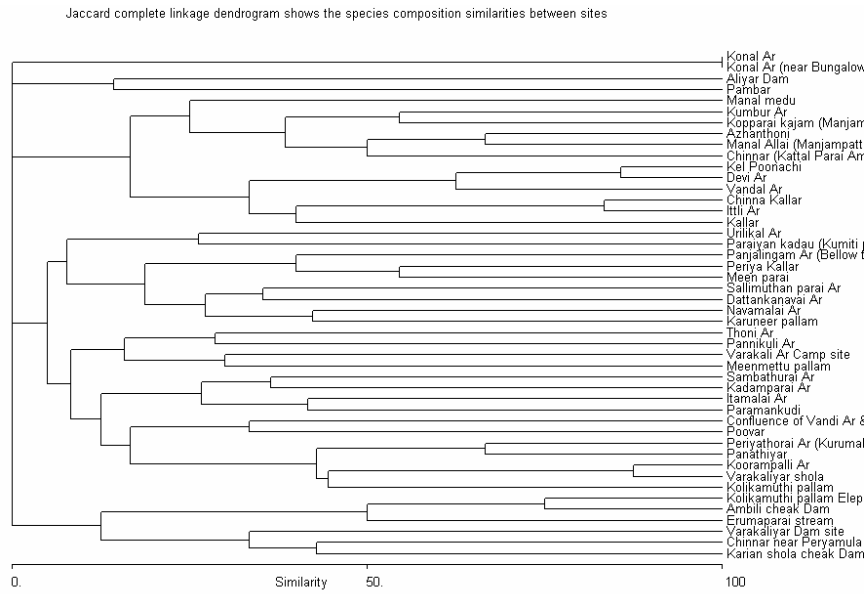
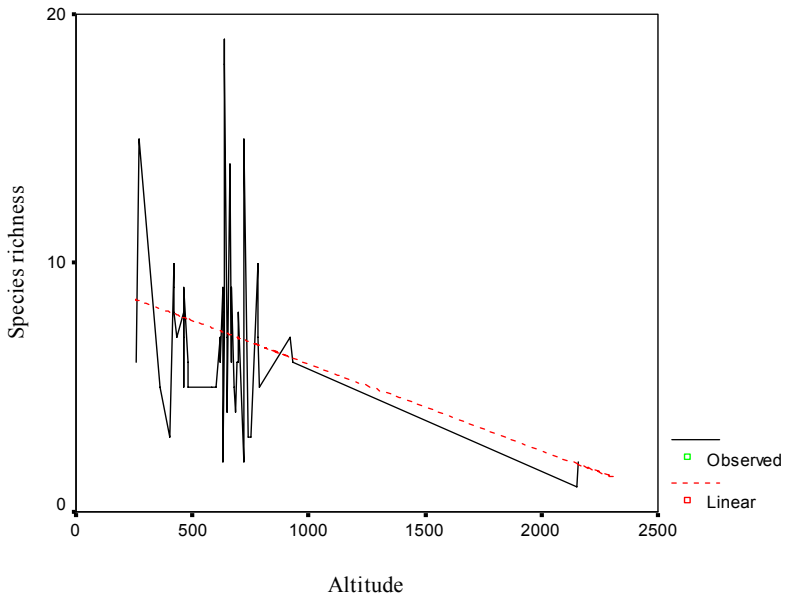


Figure 14.13: Jaccard complete linkagedendrogram shows the species similarities between sites.

14.6.8 Species Richness and Abundance in Different Dimension

Linear regression analysis showed a highly significant relationship between the altitude and species richness and abundance. Species richness and abundance were high below altitude 800 and low above 800 lower altitudes. Both species richness and abundance was strongly correlated with altitude. Species richness was high with increasing stream order and species abundance was high in lower order stream and low in higher order stream. Stream order showed a linear significant relationship with species abundance and richness. Linear regression showed a singnificant relationship between velocity and species richness and and with depth shows highly significant relationship between species richness and abundance (Figure 14.14-14.17).



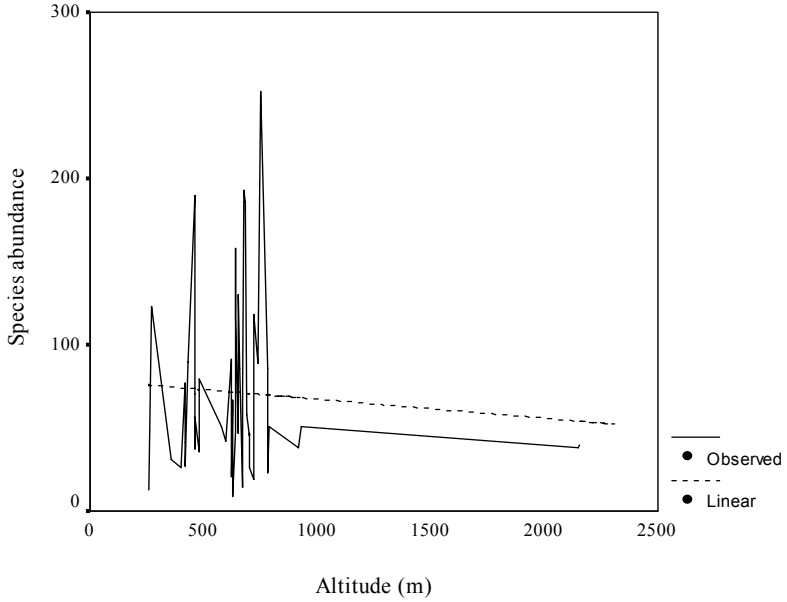
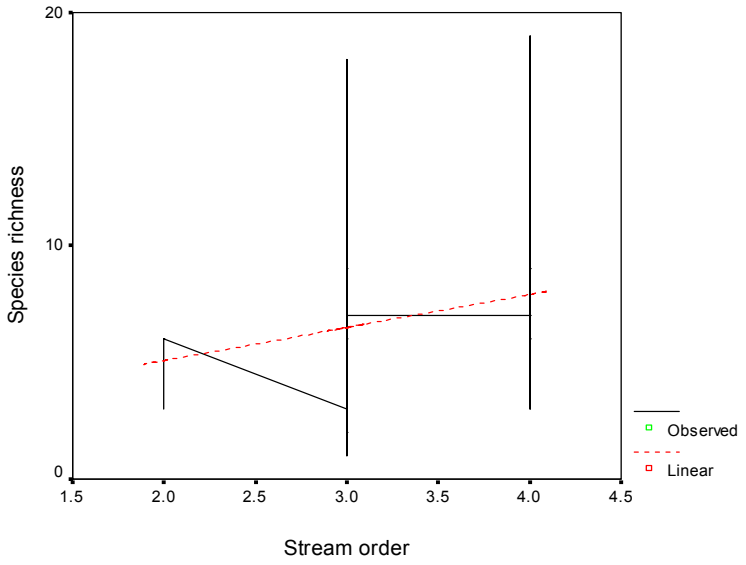


Figure 14.14: Linear regression shows the relationship between the Altitude and species ricness and abundance.



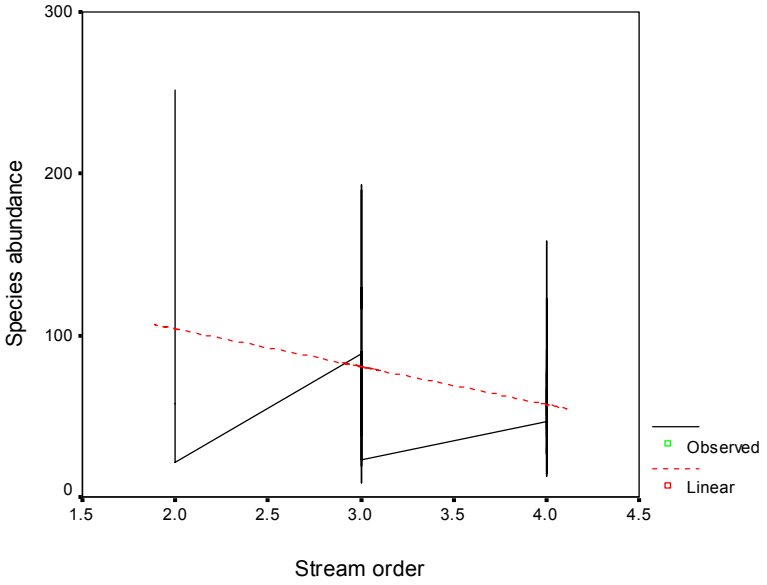
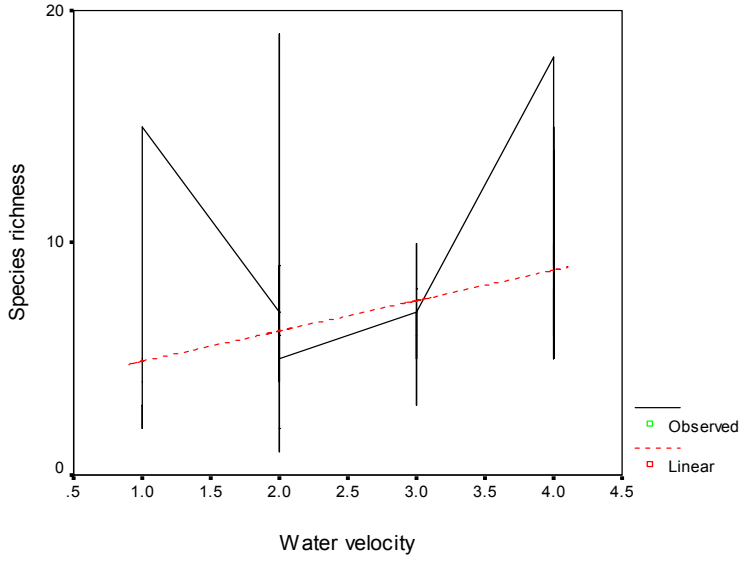


Figure 14.15: Linear regression shows the relationship between the stream order and species ricness and abundance.



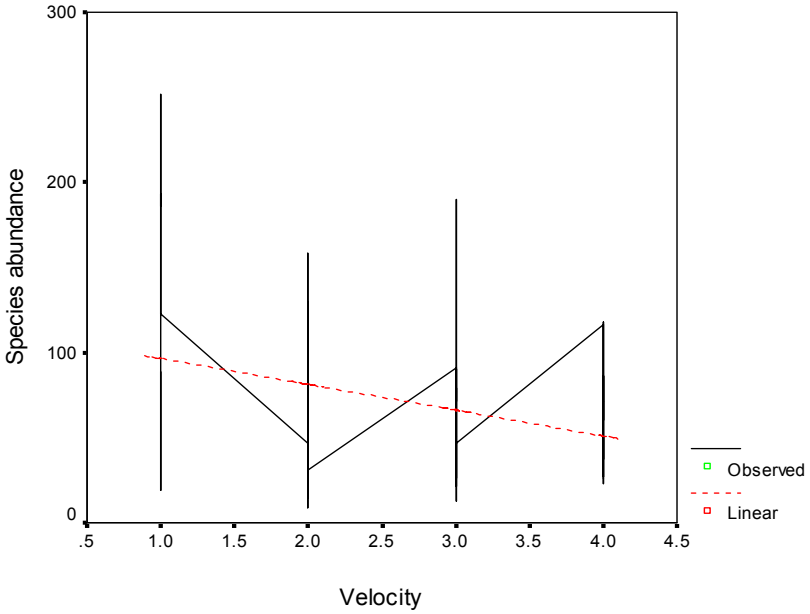
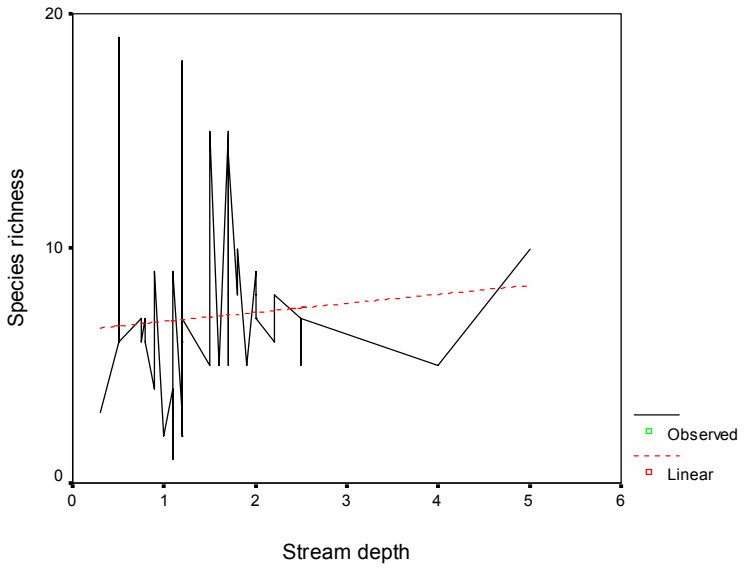


Figure 14.16: Linear regression shows the relationship between the water velocity and species ricness and abundance.



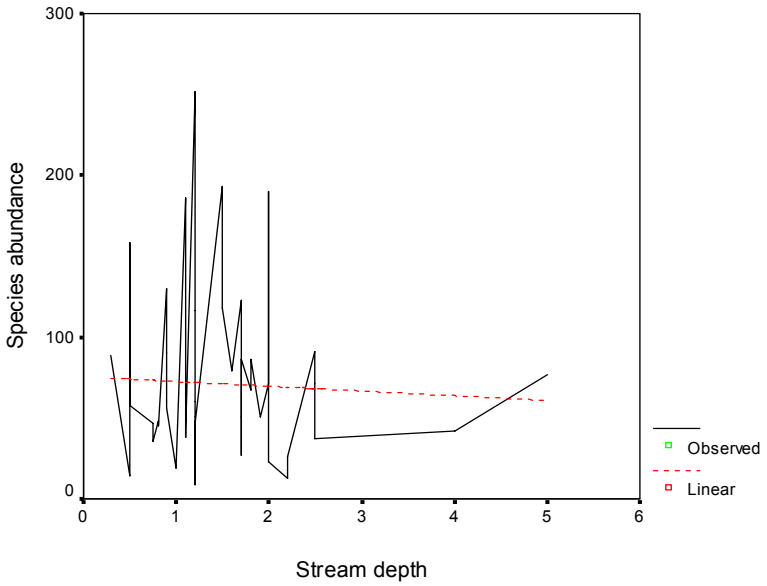


Figure 14.17: Linear regression shows the relationship between the stream depth and species ricness and abundance.

14.7 DISCUSSION

Silas (1951) listed 25 fish species from Anamalai hills and 10 species from Neliampathi hills. His study extended the distrubition of several species earlier known only from the central division of the Western Ghats to the southern division beyond the Palghat gap. Earlier work on the southern part of the Western Ghats after Day (1865, 1875-78) is by Herre (1942, 1945) who described two new species, a sisorid catfish *Glyptothorax housei* and a homolopterid fish *Homoloptera montana* and extend the distribution of *Travancoria jonesi*. Silas (1951) in his faunal account discusses the extension of range of *Salmostoma acinaces* (=*Chela argentea* Day), Barbodes carnaticus (=*Barbus* (*Puntius*) *carnaticus*), *Osteochilus* (*Osteochilichthys*) *thomassi* and *Batasio travancoria* and lists 2 endemic species described by Herre viz. *Homoloptera Montana* and *Glyptothorax housei.* His further reports 5 species from the Cochin part of the anamalai hills viz. *Barilius bakeri, Puntius denisoni, Travancoria jonesi, Noemacheilus triangularis* and *Batasio travancoria.* The species *Glyptothorax madraspatanus* reported by Silas from Anamalais has been subsequently described by him as a new species *G. anamalaiensis*.

Rema Devi *et al.* (2003) reported 59 species from Anamalai hill of which 30 species are new additions to the IGWLS and 20 to the Anamalai

hills, incuding one new species *Heteropneustes longipectoralis* (Rema Devi and Raghnathan, 1999). Sevaral species reported only from central Western Ghats have now been recorded from the southern ranges beyond the Palghat gap. The extension of range of *Noemachelius monalis* has been discussed by Indra *et al,* (1998). Other collection of significance are *Hypselobarbus dubius* first described from Bhavani river at the base of Nilgiri subsequently reported from Manimuthar river has now been collected from the Anamalai hills. *Punitus bimaculatus* (*B. puckeli*) earlier considered as a juvenile of *P. dorsalis* has been collected from these hills. Interestingly this species is found to be the most dominant *Puntius* species in the hill ranges of the Eastern Ghats especially Javadi hills. *Puntius punctatus* earlier considered as a synonym of *Punitus ticto* has been kept as a seprate species (Menon, 1999) and both these species have been collected from IGWLS. *Garra hughi* described from the cardamom and Palani hills has been collected from many places in the IGWLS sanctuary.

Diversity in the Anamalais is very high except for a few areas such as the Aliyar river basin. The lack of diversity in the Aliyar river basin is due to the fact that most of the streams in the area are non-perennial and are prone to disturbance/contamination by the local tribal people.

In the IGWLS the species diversity and richness is high in Varakali Ar camp site, Karuneer pallam and Navamalai Ar. This diversity is attributed to the controlled fishing activity by locals and protection by Forest officials. The physical environment like forest vegetation, reparian vegetation, water temperature, habitat type, and instream cover (which provide hiding places for fish) play a major role in species diversity and richness. Simpson (1964) proposed the heterogeneity theory, which holds that the more diverse the physical environment, the more complex its flora and fauna is. The greater the variation in topography relief, the more complex the structure of the fauna; similarly the more types of habitats the community contains, the more kinds of species it will hold. This theory is supported by the fact that forest with marked vertical structure holds more species. This hypothesis is true in the present study on fishes.

Altitude also plays a major role in species diversity. Colinvaux (1930) proposed the theory of diversity that changes with altitude on mountainsides – diversity is lowest at high elevation and vice versa. The present finding supports the above theory. In Anamalai, the highest species richness and abundance are at lower elevation except Karian shola cheak Dam, Erumaparai stream, Ambili cheak Dam and low at

higher elevation in grass hills. In the present study the highest species richness and abundance was noticed below 800 m. Altitude controls many factors such as temperature, forest type, water velocity and nature of the stream. In the higher altitude the nature of forest types are mostly evergreen and grassland shola. In these kinds of forests there is a domination of a single species. It is true in the present study conducted in the Grasshills stream site, the domonant plant species is grass. At higher elevations, the streams found are first and second order streams; the water temperatures of these streams are lower when compared to lower elevation streams, leading to low diversity.

Most study sites are clustered with nearby areas in the same river basin to facilitate study and comparison. When geographical proximity is not used as a parameter for clustering, habitat characters such as altitude, forest types, water temperature, substrate types and depth are considered. The species composition in these sites shows a lot of similarity. Some of the sites where human disturbances are prevalent and exotic species are present, fall in the same cluster. Some sites like Konal Ar remain separate, because only *Salmo gairdenerii gairdnerii* and *Salmo trutta fario* were present and no other species were found. There are two main reasons for this separate clustering – one, due to the rare species forms and two, two due to low water temprature. When the compositions of the fish communities at different sites were compared, it was found that there is not much similarity between the sites.

14.8 ACHIVEMENTS AND RECOMMENDATION FOR FURTHER RESEARCH

In Indira Gandhi Wildlife Sanctuary most of the research work was carried out on the large mammals and other higher vertebrates. This is the first comprahencive attempt to study the fish faunal diversity (Plate I-X). The distribution of exotic species was identified; this is very important for the conservation of fish fauna of the IGWLS. Totally 68 species were collected in one time sampling of which six species are found to be new to science. An informative colour poster depicting 68 species was printed. The endemic and threatened fishes were idendified and their habitat conditions studied. The record of five Tor species in the sanctuary is another significant finding made in the IGWLS Fifteen species have been recorded for the first time in the IGWLS.

Since this study was carried out during the summer; hence most of these streams were dried up and samples of species could not be

collected in their original habitat. It is receommended that a seasonal study is carried out for a better understanding of the fishes of the IGWLS. Ecology, biology, food and feeding ecology and breeding behaviour for many of the indigenous species are not known, further research can focus on these aspects.

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