GENETIC AND GENOMIC TOOLS IN FISHERIES AND AQUACULTURE

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Introduction

The fitness and survival of diverse organisms in a particular environment are dependent on the unique biological attributes of a species. The inherent genetic variation within species and populations provides the necessary impetus to thrive in the presence of dynamic environmental forces. Understanding the genetic and morphological diversity is the key to ensuring the sustainability of ocean resources, identifying adaptive evolution patterns and managing ocean resources. Sustainable fisheries management requires the identification of fish species accurately to preserve the biological complexity. Identification of stock structure is essential to devise management strategies specific to the stocks. Recent advances in molecular tools have enabled unambiguous identification and stock structure delineation enabling conservationists to plan management and conservation actions.

Molecular taxonomic tools

Molecular taxonomic investigations have undergone tremendous advancements in the last decade due to the advent of sequencing methods. The power and precision associated with molecular taxonomic investigations have contributed immensely to the advancement of knowledge in many areas of biological science. Several molecular markers are in use for understanding intra- and inter-specific patterns of diversity in marine populations and they can be characterized as Type I and II. When a marker is associated with genes of known function they belong to the Type I category and when associated with genes of unknown function, they belong to the Type II category.

Type I markers

Allozyme markers are type I markers as they are associated with genes of known functions. DNA encodes allozymes and genetic variation at the level of enzymes can be detected using allozyme electrophoresis. Allozymes are protein variants which originate from allelic variants which differ in electric charge and these variations can be detected using electrophoresis. Allozymes are codominant markers expressed in heterozygous individuals in a Mendelian fashion. Information regarding single locus genetic variation can be gathered using allozyme analysis which can answer many questions regarding the intra- and inter-specific diversity of fish populations. To detect variations in allozyme patterns, allozymes have to be extracted from tissues following standard protocols and variations are detected through electrophoresis in an acrylamide or cellulose acetate gel. A single band will be present if individuals are homozygous and double bands when individuals are heterozygous. Allozymes have been extensively studied and used for many investigations due to their simplicity and cost effectiveness as any kind of soluble protein can be used for allozyme analysis. Many numbers of loci can be screened at a time using allozyme markers. Major limitations with the use of allozyme analysis include the requirement of a large amount of tissue

which impedes its use with smaller organisms like larval forms. The tissue sampling method is invasive and hence every time the fish has to be sacrificed and tissue stored cryogenically. Point mutations in nucleotide sequences could not be detected using protein electrophoresis as such mutations may not result in a change in the amino acid composition. Despite all these limitations, allozymes have been widely used for fishery biology investigations like fish systematic, population genetic structure, conservation genetics and forensic applications.

Mitochondrial DNA markers

Mitochondrial DNA is found in the cytoplasm inside organelles called mitochondria and hence they are considered as non-nuclear DNA. Mitochondrial DNA can be considered as a haploid genome which is maternally inherited and transcription takes place as one single unit. Mitochondrial DNA is not subjected to recombination events, is selectively neutral and present in multiple copies in each cell. It is easy to isolate mitochondrial DNA from any tissue or blood sample as they are physically separated from the cell's DNA. Detection of population bottlenecks and hybridizations is easy using mitochondrial DNA as effective population size is smaller than nuclear DNA due to its maternal inheritance. Molecular taxonomic methods using mitochondrial DNA employ either RFLP-based length polymorphism or sequence variations to detect differences in patterns of genetic diversity. RFLP is based on the length of polymorphisms generated by digesting mitochondrial DNA with restriction enzymes and visualizing these polymorphisms electrophoretically. RFLP has been widely used to understand species-specific patterns in many marine fishes.

Sequencing the amplified region of mitochondrial DNA has become very popular now after the emergence of sequencing technologies. Several universal primers are available based on conserved sequence regions so that they can be applied universally to any tissue type. Inter-specific comparisons can be carried out using slow-evolving gene regions and intra-specific comparisons using fast-evolving gene regions. The gene, Cytochrome C Oxidase 1 (around 600bp) is being used as the universal barcode for species-specific comparisons as it is a slow-evolving region. It is highly conserved across a wide range of taxa. D-loop region, the only non-coding region is fast evolving and used for intra-specific or population comparisons. In addition, cytochrome b and ND-1 and ND-5/6 regions are also being used for intra-specific comparisons. Mitochondrial DNA genes find widespread application in fish systematics and population genetics.

Nuclear DNA markers

Nuclear DNA markers can be categorized as arbitrary and specific depending on the gene regions to be amplified.

Arbitrary markers

Arbitrary markers include; RAPD, Random Amplified Polymorphic DNA and AFLP, Amplified Fragment Length Polymorphism. RAPD makes use of an arbitrary marker to amplify regions of genomic DNA and exhibits a very high amount of polymorphism. This marker does not require any knowledge of genes or genomes so it is very fast, cheap and efficient. But RAPD markers lack reproducibility and repeatability and many products are simultaneously generated. Homozygous and heterozygous states cannot be distinguished based on these markers and slight changes in amplification conditions bring about variations in band patterns. AFLP markers employ both RFLP and RAPD techniques. Two restriction enzymes are used to digest genomic DNA followed by the ligation of double-stranded nucleotide adapters to the ends of DNA fragments which will be the primer binding sites for subsequent amplification by PCR. Primers which are complementary to the adapter and sequences at the restriction site with additional nucleotides

at the 3' end can be used as selective agents which can amplify a subset of ligated fragments. Polymorphisms along with the presence or absence of DNA fragments are detected in polyacrylamide gels.

Specific markers

A variable number of tandem repeats is parts of DNA repeated tens, hundreds or thousands of times within the nuclear genome of eukaryotes. They are repeated tandemly, varying in number at different loci of the genome and individuals. Repetitive DNA can be classified as minisatellite and microsatellite DNA. Minisatellite DNA are loci with repeats of length varying between 9-65bp and microsatellite DNA with repeats 2-8bp. Microsatellites are found abundantly in the genome as compared to minisatellites and they are widely used in population genetic analyses. Minisatellites are of two types; multilocus and single-locus. Multilocus minisatellites consist of tandem repeats of 9-65bp with lengths varying between 0.1 to 7kb. Minisatellite loci can be used for parentage analyses whereas they are less useful for population genetic analyses. Their mutation patterns are very complex and hence interpretation is very difficult for population genetic analyses. Most of the research works are concentrated on single locus minisatellite probes which are successful in detecting population genetic variations. Other applications of minisatellite loci include forensics, parentage analysis, understanding mating success and confirmation of gynogenesis.

Microsatellites are simple repeated sequences in the genome which are highly variable. These loci can be used as markers and are seen every 10kbp of the genome. They are very useful in genome mapping and population genetic investigations. These loci are highly variable, selectively neutral, and do not code for proteins and hence the amount of sequence divergence may be directly related to the time since separation. Microsatellites evolve faster at a rate of 10^{-3}-10^{-4} mutation/generation and are inherited in a Mendelian fashion. They are considered as codominant markers. Due to the high level of polymorphism, they are very popular. Cross amplification with primers developed for closely related species minimizes the cost associated with microsatellite detection and characterization. The procedure of microsatellite amplification involves extraction of DNA, amplification using specific primers and visualization of bands in PAGE gel. Automated genotyping by using labelled primers has analyzed size polymorphisms accurately and fast. The presence of null alleles and stutter bands is the major limitation while using microsatellites. Null alleles occur due to mutations at primer binding sites which will decrease accuracy in parentage or relatedness analysis so discarding loci showing null alleles is the best option. Stutter bands are formed due to slipped strands mispairing during PCR or inadequate denaturation of amplification products. Stuttering is relatively less with tri- and tetra-nucleotide repeats. Microsatellite markers can be used in fisheries and aquaculture for detecting the genetic structure of populations, conservation of biodiversity, phylogenetic investigations, phylogeographic studies, and understanding the impacts of stocking and hybridization. It can also be employed for the forensic identification of individuals, mapping of the genome, and determination of kinship and patterns of behaviour.

Single nucleotide polymorphisms arise in the genome due to single point mutations like insertions/deletions and transitions or transversions. Point mutations produce divergent alleles with alternative bases at a specific nucleotide position and these alleles are estimated to understand intra- and inter-specific diversity patterns. SNPs are considered as the most abundant polymorphism in the genome which can be detected using PCR, microarray chips or fluorescence technology. SNPs are described as next-generation markers in fisheries and can be widely applied for population genetics and genomics investigations.

DNA microarrays are small glass microscope slides, nylon membranes or silicon chips which can hold many immobilized DNA fragments in a standard pattern. A reporter probe of a known sequence can be

matched with DNA from a target sample which is of unknown origin. Microarray can also be used for the construction of species-specific DNA probes which could be subsequently used for identification purposes. DNA of the target samples has to be labelled with fluorescent molecules and hybridized to the DNA of the microarray. A fluorescent signal will be emitted when hybridization is positive which can be detected using appropriate fluorescence scanning/imaging equipment.

Expressed sequence tags can be generated using random cloning of cDNA and they can be used for identification of genes and analysis of expression using expression analysis. It is possible to make fast and reliable analyses for the genes expressed in particular tissue types under specific physiological or developmental stages. cDNA microarrays can be used to identify differentially expressed genes properly. In addition, ESTs can also be used for linkage mapping.

Molecular markers in fishery biology investigations

To understand inter and intra-specific variations

The extent of divergence in DNA or genes is considered the baseline for species-level differentiation and the variability in evolution among taxa should also be considered while making decisions. Mixed catches, larval forms, endangered and threatened animals caught illegally, stranded cetaceans and processed fish products can be identified up to the species level using molecular markers as morphological identification is not possible with these samples. Molecular markers can be used for fish stock characterization or identification of sub-species.

Phylogenetic and Phylogeograhical studies

Phylogenetic studies focus on historical processes affecting species relationships whereas phylogeographic studies focus on processes affecting geographical distribution. Mitochondrial DNA markers can be utilized effectively for phylogenetic and phylogeographic investigations. Based on mitochondrial DNA information, the evolutionary history of groups of fishes can be reconstructed and vital knowledge on historical demography obtained. In addition, conservation units and ecological patterns are also deduced. Mitochondrial DNA has been used as a powerful tool to infer intraspecific phylogenetic patterns in many marine fishes.

Identification of genetic structure between and within populations

Identification of the stock structure of fish populations is vital for fisheries management and conservation. Stocks are subpopulations within species which may be reproductively isolated and exhibiting different physiological and behavioural patterns. Mitochodrial DNA as well as microsatellite markers are widely used for inferring the genetic stock structure of marine fish populations. Morphological and meristic information should be combined with genetic information so that a comprehensive picture of subpopulation structure is obtained. Identification of subunits from mixed fisheries or the origin of stock components is also possible using molecular tools.

Genetic tagging/marking

Individual fishes can be marked for tracking movement or migration, understanding population size or contributions of distinct stock to the mixed fishery. Since physical tags are not heritable, they cannot be employed for generations. Genetic tagging by tracking a rare allele in individuals of populations over generations will be beneficial to understanding the contribution of the hatchery programme on harvest and identifying migrants from different regions.

Forensic investigation

Molecular markers are very effective in identifying dead or stranded fishes and preserved or canned items as morphological identification is not possible in them. Certification of fishery products and detection of illegal trading of fish and fishery products is also possible using molecular forensic technologies. Molecular tools also could be used for monitoring the deliberate or accidental release of fish/organisms into natural waters.

Studying the trophic relationships

Determining trophic relationships within the ecosystem is essential for any ecological study and data on diet composition is crucial towards achieving this aim. It is difficult to identify diet components up to the species level using morphological features alone as partial or complete digestion will destroy key morphological features. DNA can be extracted from partially digested samples and diet components studied using molecular markers.

Ancient DNA to deduce historical evolutionary relationships

Preserved samples in museums, fossil remains, archaeological finds and other unusual sources can be utilized for the retrieval of DNA sequence information using several methods. Several such investigations have improved our understanding of evolutionary relationships among different taxa.

Applications in aquaculture

Molecular markers can be effectively used in aquaculture for; selective breeding and genetic improvement, finding quantitative trait loci, identifying inbreeding events, genetic identification of hatchery stocks, progeny assignment, marker-assisted selection, understanding the effects of ploidy induction and gynogenesis. Molecular markers have a wide range of applications in disease diagnosis. PCR-based kits are available for the detection of many diseases like white spot syndrome virus (WSSV), infectious pancreatic necrosis virus (IPNV), viral nervous necrosis virus (VNNV), channel catfish virus (CCV), infectious hematopoietic necrosis virus (IHNV) and viral hemorrhagic septicemia virus (VHSV).

Omics in fisheries

Information from many genome-wide markers is used in population genomics in contrast to population genetics where only a few markers are used. Information regarding both genic and non-genic regions could be generated using population genomic tools and hence effects due to selection, mutation and recombination on populations could be distinguished from effects due to genetic drift, inbreeding and gene flow. When only neutral markers are used, information about adaptive evolution is missing as adaptive divergence plays a very important part in local adaptation. Local adaptation provides optimum fitness for adapting to a particular environment or habitat which is a driver for subpopulation structuring in fishes. Knowledge about locally adapted populations is also important to suggest conservation measures aimed at conserving genetic diversity at ecological time scales. Climate-related habitat shifts and movements of marine fishes can be predicted if we have information about environment-induced microevolution. Genome-level prediction of footprints of selection associated with a particular trait is not very easy as several genes are involved in determining the characteristics of an adaptive trait. Population genomics becomes more relevant in this context as genomic variations in many functional genes linked to adaptation and selection within and between populations in space and time could be delineated using genomic tools. Several methods like Expressed Sequence Tags Sequencing (EST), transcriptome sequencing and whole genome sequencing will help in inferring important information regarding functional gene modifications linked to environmental alterations.

Approaches in genomics

Studying the variations in the expression of functional genes which have important roles in adaptation is one of the best approaches to understanding variations in real time, which is otherwise known as the candidate gene approach. Functional genes having a known function and influence on an adaptive trait can be selected for these studies. Genes which are linked to variations in the structure of fishes or related to physiological processes and consequent phenotypic variations can be selected. Sequence information or allele frequency-based tests can be used to derive information regarding functional gene variations. The action of evolutionary forces can be understood based on sequence data or allele frequency-based tests. The candidate gene approach is relatively faster as comparative genomic approaches using candidate genes enable efficient characterization and comparison of different genes and a controlled experimental set-up is required only if a direct linking with phenotypic variation is desired. Efficient detection of variations across populations could be carried out which will help in understanding the influence of selective pressures concerning environmental fluctuations. The major challenge in the candidate gene approach is to identify the correct candidate gene related to the trait under study and the time-consuming sequencing to understand variations in functional gene expressions.

In the genome scan approach, regions or loci exhibiting a high amount of structuring due to the effect of genetic hitchhiking are studied. Balancing selection or direction selection will bring about variable patterns on such loci and hence studying those loci will provide insights into selective forces acting on the genome. A large number of genetic markers widespread in the genome like microsatellites, SNPs, AFLPs or functional markers like genes linked to ESTs could be employed for this method. Transcriptome sequencing could be used to detect genes linked to ESTs. Thus, genome scan approaches help in detecting loci under selection. QTL mapping relies on information from linkage maps which are constructed using a dense set of markers providing clues concerning the genetic basis which determines phenotypic traits and this is achieved through family crosses under controlled conditions in the laboratory. QTL information could be compared among closely related species minimizing the cost associated with it. Since QTL investigations require controlled experimental conditions, species amenable to aquaculture will be the best candidates for QTL-related experiments. Association mapping is based on the information regarding linkage disequilibrium between large numbers of genetic markers which is linked to phenotypic variations. Admixture mapping helps in detecting natural events of admixture like intra-specific hybrid zones and we make use of the information regarding linkage disequilibrium to map admixture zones in the wild.

Seascape or landscape genomics tries to correlate information from genetic markers with geographic information for deriving insights regarding geographic patterns of genetic differentiation. Both neutral and non-neutral or functional gene markers can be employed in seascape genomics so that selection information can be deduced based on the divergence level of functional genes at each geographic location. Genetic divergence in non-neutral markers if any is indicative of locally adapted populations.

Data regarding differential gene expression patterns are very important to understanding the role of key functional genes in modulating vital physiological processes. Transcriptomic approaches like mass-scale RNA sequencing are very useful to understand variations in gene expression patterns in real time and these methods can be applied to all species due to the ease of transcription profiling and annotation. Micro-arrays can be used to measure the differential gene expression of hundreds or thousands of genes simultaneously whereas real-time PCR measure the gene expression of one or a few candidate genes.

Real-time PCR is based on the principle of normal PCR and the product is measured in real time by tagging the reaction mixture with fluorescent dyes. Fluorescence can be measured after each PCR cycle

which corresponds to the expression levels of a gene. The main drawback of real-time PCR is that only very few genes within a genome can be studied using this method. However, microarrays enable the investigation of gene expression of thousands of genes simultaneously. Microarrays consist of thousands of single-stranded DNA spots attached to surfaces like glass slides with each spot corresponding to a gene. Gene expression can be measured by washing fluorescently tagged cDNA over the slides so that complementary strands hybridize on to the array and fluoresce. The intensity of each spot will be proportional to the expression of that particular gene. All these investigations require controlled experimental conditions to relate gene expression data with adaptive variation.

Conclusion

Genetic and genomic tools can be effectively employed to enhance fisheries and aquaculture productivity and ensure sustainability. Markers should be selected cautiously to improve the quality of the output. Next Generation Sequencing technologies are revolutionizing the field of biology and the future of aquaculture and fisheries will depend on the penetration of these technologies to this vibrant sector.

Suggested reading

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CAPTIVE BREEDING AND LARVAL REARING OF MARINE FISHES

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Introduction

Breeding and seed production of marine fishes depends extensively on quality broodstocks, induced maturation, spawning, larval rearing, live feed culture, and the availability of good quality water. A combination of water temperature control and photoperiod regulation in a recirculation system could further speed up the maturity. It may prove helpful in a commercial broodstock development system (Gopakumar *et al.,* 2012). Following the successful seed production of Cobia, Silver pompano, Indian pompano, Vermiculated spine foot, Pink ear emperor, Black seabream, Orange-spotted grouper and John's snapper, the farming demonstration of those species in marine cages and brackish water cages/ponds popularized the technology among the farmers about its suitability for aquaculture.

Cobia (*Rachycentron canadum*)

The Mandapam Regional Centre of CMFRI initiated research on the seed production of cobia during the year 2008. The Centre has succeeded in developing protocols for captive breeding, seed production and cage farming of cobia. Sub-adult cobia were collected from the wild and stocked in sea cages for development as broodstock. They were fed with squids, oil sardines and lesser sardines with vitamin premixes. The fishes were cannulated and sexed. PIT (Passive Integrated Transponder) tagging was done for identification of the brooder. It is a radio frequency device to permanently mark the fish internally. The PIT tag contains a microprocessor chip and antenna. The implant site depends upon the species, the size of the fish and the size of the tag. It is preferable to implant the tag on the dorsal musculature of the fish which will be convenient for the brood fishes to be read. Fishes weighing around 9 kg and above, were transferred and stocked in 60 t capacity FRP tanks/100 t capacity cement tanks with a recirculation system in an on-shore hatchery facility at the male: female ratio of 2:1. Then, these fishes were provided with special maturation diets viz., squids, cuttlefish, crab, shrimps and chopped oil sardines once in a day. Usage of different hormones namely Luteinizing Hormone Releasing hormone (LHRHa) and Human Chorionic Gonadotropin (HCG) were studied at different dosage levels to standardize the optimum dosage. Once the ova reached a size of 700 µm diameter, the females were induced with HCG at the dose of 500IU/Kg body weight. The males are administered a dosage of 250IU/Kg body weight. The spawning occurred within 36 hours after injection. The number of eggs spawned by cobia ranges from 0.4 to 2.5 million.

Cobia (*Rachycentron canadum*)

The fertilized eggs which were floating at the surface were collected by using a 500 µ mesh and incubated. The unfertilized eggs which settle at the bottom were removed by siphoning. The fertilized eggs were incubated in 2-tonne capacity rectangular/circular tanks. Stocking density can be maintained at a moderate level of 200 to 500 eggs per litre. The development of the embryo can be observed at frequent intervals under a stereo/compound binocular microscope. The hatching took place between 18 to 22 hours. Before stocking the newly hatched larvae into larviculture tanks, at least 10 to 20 larvae were checked under a microscope for deformation/abnormalities, pigmentation and appearance of internal organs. The newly hatched cobia larvae measure around 3.4 mm and were stocked in 2-tonne capacity tanks containing filtered seawater at a stocking density of 5-10 nos./ L. The tanks were provided with mild aeration and microalgae at a density of $1x10^7$ nos./ mL. Special care is needed when selecting microalgae for growing live feeds for marine fish larvae, to avoid the nutritional deficiencies of the latter, especially in terms of n-3 highly unsaturated fatty acids. Deficiencies in the n-3 PUFA contents of microalgae may cause severe mortalities and quality problems in marine fish larvae. The mouth of the cobia larvae opens on 3rd day and the mouth size was around 230 µm. Newly hatched cobia larvae generally started feeding at 3 dph and they can be fed with the enriched rotifer (*Brachionus rotundiformis*) at the rate of 10-12 nos./mL, two times a day till 10 dph. From 8 dph, the larvae can be fed with enriched Artemia nauplii at the rate of 5-6 nos./mL, 2 times a day. During the rotifer and Artemia feeding stage, the green water technique can be used in the larviculture system with the microalgae *Nannocloropsis occulata* at the cell density of $1x10^7$ cells/mL. The Artemia nauplii were provided at a density of 5-6 nos./mL up to the 19th day. Weaning to larval inert feed was started from the 15th day and co-feeding with Artemia was continued till the 19th day. While weaning, formulated feed was given 30 minutes before feeding with live feed. Frequent grading is needed from this stage to avoid/reduce cannibalism. Everyday grading is advisable for better survival. In addition, a variety of other factors such as tank colour, size of the tank, water temperature, water quality, etc., may also affect larval survival and growth. From 20th day, the feeding was entirely on inert larval feeds. The size of the artificial feed has to be smaller than the mouth size of the fish. Continuous water exchange was required during the weaning stage. The metamorphosis of the larvae started on the 18th day and all the larvae metamorphose into juveniles by the 21st day. The water exchange is practically nil till 7th day and it can be gradually increased from 10-100 % from the 8th to the 25th day after hatching. The environmental conditions required during the larviculture period were DO: > 5mg / L; NH3: < 0.1mg / L; pH: 7.8 – 8.4; salinity: 25-35 ppt and water temperature: 27-33° C. Nursery rearing was carried out from 25-55th day. During this stage, the fingerlings were initially provided with an artificial feed of 800µm size. Thereafter, the fingerlings were fed with a progressively higher size range of floating extruded larval feeds. A daily water exchange of 100% is advisable. Water quality parameters like salinity, temperature, pH, oxygen level and ammonia were closely monitored during the entire larviculture period. After the 55th day after hatching, fingerlings with sizes ranging from 3-4 inches can be supplied to farmers for stocking in the sea cages/ponds for further nursery rearing and grow-out farming.

Indian Pompano

The common name or FAO name of *Trachinotus mookalee* is "Indian pompano". *T. mookalee* belongs to the family Carangidae (jacks and pompanos) and holds immense potential for the marine finfish aquaculture sector. It is a pelagic species which inhabits shallow coastal waters. Indian pompano is most common in shallow coastal waters in several environments, including coral and rocky reefs, shore faces and tidal flats. The species has a wide salinity tolerance, as evident from the ranges from which juvenile and sub-adult fish are caught in Indian waters.

Indian Pompano (*Trachinotus mookalee*)

Initially, Indian pompano broodstock can be developed by collecting or purchasing wild fish. Adult fish of more than 3 Kg can be collected from the wild by hook and line and transported to the hatchery. The sexes are separate and the maturation size is around 3.0 Kg, thus the same size fish needs to be collected in more numbers for developing broodstock. Once the fish are transported to the hatchery, the fish are immediately to be shifted to the quarantine area. It is to reduce the parasitic or bacterial infection. Generally, the quarantine period varies from 3 to 4 weeks and can be carried out in a small tank of 1 m^3 size to facilitate easy handling. Generally, broodstock tanks are used for the culture and maturation of broodstock as well as for spawning. Due to the size of the broodstock and the natural behaviour of the fish, larger tanks of 50 -100 m^3 are preferred for maintaining the broodstock. At Visakhapatnam Regional Center of ICAR-CMFRI, 125 m^3 concrete reinforced tanks with a water volume of 100 m^3 are used for broodstock development cum spawning tank. Broodstock tanks should have re-circulating facilities with 300% water re-circulation. Sea water used for broodstock development should be filtered and clear with stable salinity of 30-35 ppt and water temperature of 27-32°C. Tanks should have a natural photoperiod. Indian pompano needs to be stimulated with the use of hCG hormone for spawning. Once the female ova size attained a size of more than 500 µm, and the males are oozing, then both the sex need to be injected hCG at the rate of 350IU/ Kg body weight. The fish for cannulation should be anaesthetized to avoid stress during the cannulation and injection. As far as possible, avoid the checking of males before inducing for spawning. Then the fish are shifted back to the same tank for spawning. The fishes respond after 36 h of injection at a temperature range of 28-32°C. The re-circulating system is to be stopped by 7.00 pm to avoid washing out eggs. Generally, the Indian pompano will respond at night between 11.00 to 12.00 pm.

The fertilized eggs of Indian pompano are floating in nature at a salinity of more than 30 ppt. This floating nature of eggs helps collect eggs by overflowing eggs in the collecting chamber. The fertilized eggs of Indian pompano are non-adhesive and pelagic and range from 0.9 to 1.0 mm in diameter. A 500 µm mesh size bag is tied in an egg collecting chamber, which is connected to a broodstock tank via a 3 inch PVC pipe. Generally, Indian pompano eggs are stocked at the eyed stage because they are more robust than the newly hatched larvae, at this stage. Newly hatched larvae are very sensitive to physical shock or changes in water quality and moving them to the larval-rearing tanks may result in high levels of mortality. The seawater used in larval-rearing tanks is pre-treated through a sand filter to remove particulate matter and is then ozone-sterilized to eliminate pathogens. The recommended initial stocking density for Indian pompano is 10 larvae/L. Live feeds used for larval rearing comprise microalgae (*Nannochloropsis* sp. and *Isochrysis* sp.), copepod nauplii and adult, small rotifers (*Brachionus rotundiformis*), large rotifers (*Brachionous plicatilis*) and brine shrimp (*Artemia*) nauplii.

The yolk sac (endogenous source) continues as the sole source of nutrients for the developing embryos immediately after hatching. The endogenous source provides nutrients for 2 days in Indian pompano larvae. Then, the exogenous feeding starts when the mouth opens after 2nd day. Their initial mouth gape is around 230 µm and hence the appropriate size of feed i.e. copepod nauplii and screened rotifers needs to be provided. *Nannochloropsis* sp and *Isochrysis* sp in the ratio of 1:3 are introduced into the larval-rearing tanks on the 2nd Day of Post Hatched (DPH) at an algal cell density of 1 x 10^5 cells/mL. Rotifers

filtered with 100 µm mesh and copepod nauplii filtered with 100 µm mesh are introduced into the larval rearing tanks on 2nd DPH, after the larval mouth opening has been formed. The rotifer and copepod nauplii density in the larval-rearing tanks is maintained at 10-15 and 2-3 individuals/mL during the 2nd -5th DPH. After 5th DPH, rotifers are introduced at densities of 20 individuals/ mL, which is gradually increased to 30 individuals/mL from 8th to 10th DPH. Rotifer density gradually decreases with an increase in the rate of rotifer consumption by the larvae and eventually by 13th DPH, the rotifers disappear. Freshly hatched out Artemia nauplii are fed at a density of 0.5 individual/mL from 8th DPH and their size increases with advancement in the rearing period. Weaning of pompano larvae with artificial diets started from 11th DPH. An artificial diet with a particle size of 200-300 µm is used initially. The formulated feed is sprinkled onto the surface of the water in small amounts frequently throughout the day. Formulated feed is added in small amounts so that the feed is consumed within 5 or 10 minutes, as excess feed should not be allowed to accumulate on the bottom of the tank where it decomposes and degrades the water quality. The size of particulate feed is increased to 400–800µm from 22nd DPH. High-quality micro diets, specifically formulated for marine finfish, should be used and these should be stored in a refrigerator or freezer to maintain their quality. Water exchange should be increased to 20% per day when both rotifers and Artemia are being fed together (8th DPH). Water exchange gradually increased to 50% per day from 11th DPH, and is 100 % per day from 16th DPH. It is of utmost importance to measure water quality regularly in the larval rearing tanks (Table 11.1). If water quality degrades, it should be necessary to exchange the water at rates higher than the rates recommended above. However, replaced water should be of similar temperature and salinity to the water in the rearing tanks to avoid stress to the larvae.

Orange Spotted Grouper

The common name or FAO name of *Epinephelus coioides* is "orange spotted grouper". The orange spotted grouper *Epinephelus coioides* occurs in the western Indian Ocean from the southern Red Sea to Durban (South Africa) and east to the western Pacific where it is distributed from Ryukyu Islands (Japan) to New South Wales; Oceania only to Palau in the Northern Hemisphere and Fiji in the Southern; and the eastern Mediterranean. In India, this species is distributed all along the Indian coast from Gujarat to West Bengal, including the Andaman and Nicobar Islands.

Orange spotted grouper (*Epinephelus coioides)*

Groupers are demersal fish and hence after catching, the fish from the wild shows barotraumas. Barotrauma occurs due to the differences in the pressure from where they are caught and the sea surface. Due to pressure differences, the air bladder fills with air and bulges and gives stress to the internal organs. This needs to be relieved by inserting a needle into the gas bladder through the anus. Once the fish are swimming normally with controlled buoyancy to maintain position in the water column, they need to be shifted to the quarantine area. It is advisable to quarantine them to reduce the parasitic or bacterial infection. Generally, the quarantine period varies from 1 to 2 weeks and can be carried out in small tanks of 1 m^3 capacity to facilitate easy handling. During the quarantine period, broodstock management should focus on reducing the parasite load of the fish by giving bath treatment in formalin at the rate of 200 mg L^{-1} for 30 min followed by 5 min dip in freshwater. Fish should be shifted to another tank after the treatment with fresh seawater. Generally, broodstock tanks are used for the culture and maturation of broodstock as

well as for spawning. Due to the size of the broodstock and the natural spawning behaviour of the fish, larger tanks of 50 -100 m^3 are preferred for broodstock tanks. At Visakhapatnam Regional Centre of ICAR-CMFRI, 125 m^3 concrete reinforced tanks with a water volume of 100 m^3 are used for broodstock development cum spawning. Generally, tanks should be round, square or rectangular with rounded corners, however round tank is preferable. The medium-range blue, green or grey colour is preferable for the broodstock tank. Tanks should be at least 2.0 m deep and preferably more than 2.5 m to allow sufficient room for the spawning behaviour of orange-spotted grouper, which involves pairs or groups of fish swimming upward from the tank bottom while releasing ova and sperm. Generally, the broodstock tanks are used as a flow-through system, however, re-circulating aquaculture systems are better for broodstock development and spawning. Sea water used for broodstock development should be filtered and clear with stable salinity of 30-35 ppt and water temperature of 27-32ºC. Tanks should have a natural photoperiod.

Orange spotted grouper are diandric protogynous, and getting male from the wild is very difficult thus a hatchery manager needs to manipulate the sex by using hormones and enzymes. Fifty per cent of the stocked and acclimatized fishes should be implanted with a pellet containing 17 á-methyl testosterone and letrazole at the rate of 5mg Kg^{-1} and 0.2 mg Kg^{-1} body weight respectively. The pellet can be prepared using gum acacia, cholesterol and 17 á-methyl testosterone in the ratio of 1:2:1. These chemical needs to be weighed accordingly and mixed with a few drops of water, which form dough and prepared in the required size as per the implanter. This prepared pellet needs to be implanted on the dorsal side of the brooders below the dorsal fin in the musculature. Before implantation, fish should be anaesthetized to avoid handling stress. This hormonal dose will convert the female to a male within 2 months. Generally round and rectangular tanks are used for larval rearing. At Visakhapatnam RC of CMFRI, a round 2 m^3 tank with 1.2 m depth is used for larval rearing. A single aeration point in the middle of the tank was provided, which was connected to either an oxygen cylinder or PSA oxygen concentrator. The aeration should be mild during the early stages (at least up to 10 days) of larval rearing to avoid physical damage to the larvae. However, it is increased gradually with the progression of the larval-rearing cycle, as the larvae become more robust. The tank should be cleaned with either liquid bleach or acid wash and dry at least for two days before stocking.

The seawater used in larval-rearing tanks is pre-treated through a sand filter to remove particulate matter and is then ozone-sterilized to eliminate pathogens. The recommended initial stocking density for orange-spotted grouper is 10 larvae/L. Oil is generally added to form a thin film on the water surface (around 0.2 mL/m^2) during 1^{st}-4^{th} DPH for preventing surface aggregation mortality in the early stage of grouper larvae. Live feeds like microalgae (*Nannochloropsis* sp. and *Isochrysis* sp.), copepod nauplii and adult, small rotifers *(Brachionus rotundiformis*), large rotifers (*Brachionous plicatilis*) and brine shrimp (Artemia) nauplii are used for larval rearing The yolk sac (endogenous source) continues as the sole source of nutrients for the developing embryos immediately after hatching. The endogenous source provides nutrients for 2-3 days in grouper larvae. Then, the exogenous feeding starts when the mouth opens after 3rd day. Their initial mouth gape is very less so they have to be provided with appropriate sizes of feed i.e. copepod nauplii and screened rotifers. *Nannochloropsis* sp is introduced into the larval-rearing tanks on 2nd DPH at an algal cell density of 1 x 105 cells/mL. Rotifers filtered with 80 µm mesh and copepod nauplii filtered with 100 µm mesh are introduced into the larval rearing tanks on 2^{nd} DPH, after the larval mouth opening has been formed. The rotifer and copepod nauplii density in the larval-rearing tanks is maintained at 5-7 and 2-3 individuals/mL respectively during the 2^{nd} -5^{th} DPH. After the 5^{th} DPH, small rotifers (filtered with 150 µm mesh) are introduced at densities of 10-15 individuals/mL, which is gradually increased to 20

individuals/mL from the 11th to the 18th DPH. Rotifer density gradually decreases with an increase in the rate of rotifer consumption by the larvae and eventually by 30th DPH, the rotifers disappear. Freshly hatched out Artemia nauplii are fed at a density of 0.5 individual/mL from 17th DPH and their size increases with advancing in the rearing period. Adult copepods are fed during the 16th-20th DPH in larval rearing. Weaning of grouper larvae with artificial diets starts from the 20th DPH. An artificial diet with a particle size of 200-300 µm is used initially. The formulated feed is sprinkled onto the surface of the water in small amounts frequently throughout the day. Formulated feed is added in small amounts so that the feed is consumed within 5 or 10 minutes, as excess feed should not be allowed to accumulate on the bottom of the tank where it gets decomposed and degrades water quality. The size of particulate feed is increased to 400–800 µm from 30th-45th DPH. High-quality micro diets, specifically formulated for marine finfish, should be used and these should be stored in a refrigerator or freezer to maintain their quality. In addition, minced fresh fish meat is fed from 30th DPH. It is very important to measure the water quality parameters regularly in the larval-rearing tanks. If water quality degrades, it should be necessary to exchange the water at rates higher than the rates recommended above. However, to avoid stress to the larvae, replaced water should have equal temperature and salinity of the rearing tanks.

John's snapper (*Lutjanus johnii*)

ICAR-Central Marine Fisheries Research Institute (CMFRI) has succeeded in developing the seed production technology for John's snapper (*Lutjanus johnii*) first ever in India. Success in captive spawning and seed production of the fish, which was carried out at the Visakhapatnam Regional Centre of ICAR-CMFRI, has set a stage for farming John's snapper using hatchery-produced seeds. John's snapper, belonging to the family Lutjanidae, is widely distributed in the Indo-west Pacific, extending from east Africa to Fiji, north to the Ryukyu Islands and south to Australia. In India, the fish is reported from both the west and the east coasts. It inhabits mostly the coral reefs and rocks, deep seas, and occasionally in estuaries. The species has immense potential for mariculture owing to its fast growth rate, easy adaptability to culture conditions, quick acceptance of artificial feed, pleasant appearance, good meat quality and high consumer preference

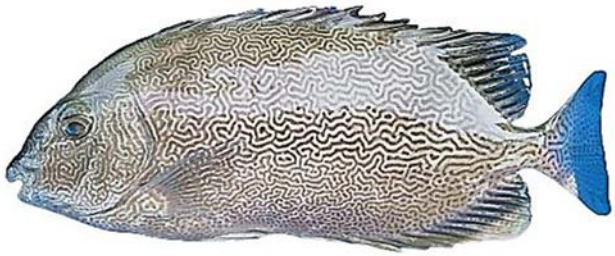
John's Snapper (*Lutjanus johnii)*

The research work was initiated with wild-caught adult fishes during 2018-19 as a component of a Department of Biotechnology-funded project entitled 'Developing a New Candidate Species for Mariculture: Marine Finfish John's Snapper, *Lutjanus johnii***.** Adult fishes of 3-3.5 kg maintained in the offshore cages installed off Visakhapatnam were selected and transported to the hatchery complex. They were subjected to prophylactic treatment for a period of two weeks and then stocked in an indigenously developed Re-circulating Aquaculture System (RAS) with a capacity of 125 t. The fishes were acclimatized and were fed upon squid, twice a day till satiation. The gonadal profile of the fish was assessed routinely using live ovarian biopsy. Once the ova size of females was found to be optimum, females and oozing males were injected with inducing hormones for spawning. Induced fishes responded 42 hours post-induction, and the fertilized eggs obtained were collected using flow-through cum recirculation of tank water. The collected fertilized eggs were treated and stocked in FRP tanks with a capacity of 2 t for incubation and larval rearing. Larvae hatched out after 14 hrs. at 28-30 °C. Mouth opened 54 hrs. post-hatching. The larval rearing was carried out using a green water system with different

live feeds such as *Nannochloropsis* sp., *Isochrysis* sp., copepod nauplii, rotifers and *Artemia* nauplii. Larvae were weaned on artificial feed from 20 days post-hatch (DPH). Larvae started metamorphosis from 22 DPH, which was completed by 30th DPH, by this time; the larvae were fully weaned on artificial feed. After 42 days of rearing post-hatch, a survival rate of 3.67% was achieved and the fry reached an average size of 3.8 cm and 0.62 g. This is the first report of successful broodstock development, induced breeding and seed production of John's snapper (*Lutjanus johnii*) under confinement in the country.

Vermiculated spinefoot (*Siganus vermiculatus*)

Rabbit fishes are widely distributed in the Indo-West Pacific region and the Mediterranean region. Inhabiting estuarine waters to coral reefs. 29 species of rabbit fishes described are worldwide. Rabbit fishes are an excellent candidate species for culture in marine and brackish water because of their herbivorous/ omnivorous feeding habits, low protein requirements, tolerance to a wide range of environmental conditions, rapid growth, consumer preference and economic value. Vermiculated spinefoot, *Siganus vermiculatus* has been reported to be one of the fastest growing species among the genus Siganus. Fishes can be collected from the wild and broodstock development could be carried out in either 6m diameter cages or 5 t RAS



Vermiculated spinefoot, (*Siganus vermiculatus)*

The fishes were fed with a maturation diet ie., 1 kg of formulated floating pellets (Growel, India; 40% protein, 10% fat, 3.5% fibre) enriched by adding 2 g of vitamin E, 1 g of vitamin C, 8 mL of cod liver oil and 20 mL of soy lecithin, at the rate of their 2% body weight twice per day for 4 months. The maturation diet was supplemented with squid meat @ 2% of their body weight during the entire rearing period. From the broodstock development facility, female brooders were identified by cannulation using a flexible catheter (2 mm inner diameter; Ramsons Scientific and Surgical Industries Pvt. Ltd., New Delhi, India) and male brooders were identified by slight pressure on the abdomen. Among the mature fishes brought to the hatchery, a female having an average ova diameter of 400 ìm and a running male were selected and stocked in separate FRP tanks. These could be induced with human chorionic gonadotropin (hCG) (two intramuscular injections @ 500 IU/ fish and 200 IU/ fish for female and male at 24 h interval respectively) or LHRH-a (two intramuscular injections @ 20 µg/kg for female at 24 h interval and single dose of 20 µg/ kg to male during last injection to female) during all phases of lunar cycle. Natural spawning without inducement can also be achieved by providing good nutrition and water quality parameters. Since the eggs are demersal and adhesive, tiles should be provided in the spawning tank for attachment and if required shifting to incubation tanks. Spawning occurs between 20 and 21 hrs after the second injection. Green water systems with microalgae, *Nannochloropsis salina* and *Isochrysis galbana* at a concentration of 2–3 × 10^6 cells/mL and

2–3 × 10^5 cells/mL were used in tanks (1000L) for egg incubation and larval rearing respectively. The fertilised eggs (0.57 ± 0.02 mm) hatched out between 24 and 25 hrs and the newly hatched larvae measured 1.92 ± 0.08 mm in total length with a transparent yolk sac (0.63 ± 0.04 mm) and an oil globule (0.23 ± 0.02 mm). A mouth gape of 89.03 ± 9.4 ìm was observed after 42 hrs post-hatch. Since the mouth gape of rabbit fish is very small, the use of copepod, *Parvocalanus crassirostris* nauplii (40-60 µm) as the first feed of larvae ensures better growth and survival. Enriched rotifers (5–37 dph), copepodites (10–20 dph), umbrella

stage of Artemia (18–24 dph), artificial pellet feeds (21–37 dph) and enriched Artemia nauplii (29–37 dph) were also used during various stages of larviculture. Metamorphosis was completed within 35–37 dph, during which post larvae with vermiculated body patterns measured 25.2–28.4 mm in length and 0.28–0.30 g in weight.

Pink ear emperor, *Lethrinus lentjan*

Lethrinus lentjan (Lacepede, 1802), often known as the pink ear emperor, is a significant food fish in Southeast Asia, the Arabian Gulf, and India. It is a member of the Lethrinidae family. Water quality parameters in the broodstock tank during the rearing period were as follows: temperature: 28–29.5/ °C, salinity: 32–35.5/ ppt, pH: 7.6–8.1, total ammonia: 0.354–0.422/ ppm, DO: 4.04–5.12/ mg/L, alkalinity: 102–122/ mg/L CaCO3; CO2: 4.12–4.67/ mg/L; turbidity: 0.16–0.39 NTU, nitrate(NO3): 0.01–0.045/ mg/L, nitrite:0.008–0.027/ mg/L and photoperiod: 12/ h/ L: 12/ h D. The fertilized eggs (714.21/ ±/ 11.91/ ìm) had a single oil globule (146.63/ ±/ 3.51/ ìm) and were clear, pelagic, and non-adhesive.

Fig. 6. Pink ear emperor (*Lethrinus lentjan*)

The raising of larvae was done using a green water system. The hatchling's size varied from 1355.1/ ìm to 1534.6/ ìm, and on the second day, its mouth opened (110–148.2/ ìm). Using copepods, rotifer, artemia, and micro diet, larvae were raised. By 22 dph, squamation and extreme body colouring had begun, and by 25–30 dph, larvae began transitioning from the pelagic to the benthic domain. Larvae became juvenile (length – 19.2/ mm; weight – 0.096/ g) after 35 dph resembling the adult fish colourations and by 46 dph, they attained an average body length of 27.83/ mm and average body weight of 0.276/ g.

Picnic seabream or black seabream (*Acanthopagrus berda*)

Picnic seabream or black seabream, *Acanthopagrus berda* is an important fish in fisheries and aquaculture because of its high recreational value, excellent meat quality, high economic value, ability to tolerate wide variations in environmental parameters such as salinity, temperature, strong resistance to diseases and faster growth rate. Therefore, *A. berda* in tropical Indian waters has the potential to attract commercial interest shortly.

Black seabream is carnivorous in nature and is a Protandrous hermaphrodite. It is a schooling species feeding mainly on echinoderms, worms, crustaceans, molluscs and small fishes. The fishes of the genus *Acanthopagrus* have good consumer acceptability due to their excellent meat quality. The approximate price for the fish in the local market in India is Rs.470/ Kg. (US $ 7/Kg).

Acanthopagrus berda

Fishes collected from the Karwar region were reared in the Marine cage farm of the Station providing maturation diets and regular ovarian biopsy was carried out to select matured fishes for the breeding programme. Matured fishes were available from September to March and females with ripe ovaries and males with oozing milt were

shifted to the Marine hatchery complex for the breeding programme.

Standardisation of breeding and seed production of this species has been achieved for the first time in India at the Karwar during February 2021, employing induced breeding techniques using Salmon GnRH – Analogue hormone (OVAFISH, Bhoomi Aqua International, India) as the inducing agent. The fishes spawned after 36 hours of inducement and the pelagic eggs hatched after 22 to 24 hours at a temperature of 28 to 30 ^{0}C. Fecundity was 0.25 million per female (450 g) and 86% of the eggs hatched after 24 hrs. Experimental larval rearing was carried out in 100 L tanks with various live feed organisms such as Copepods, Rotifers and Artemia; stocking 100 eggs /L. Metamorphosis of the larvae initiated in 24th-day post-hatch (DPH) with 9 % survival in a tank. This is the first report of the breeding of a seabream from Indian waters.

Silver Pompano

Pompanos are marine fishes in the genus *Trachinotus and* belongs to the family Carangidae. The genus *Trachinotus*, occurs in all tropical oceans and comprises 19 species (Fricke *et al.*, 2019). *Trachinotus blochii* is a deep-bodied carangid usually found around shallow coastal waters over rocky and coral reefs. Occasionally they are in small schools. The diet consists mainly of bivalve molluscs and other hard-shelled invertebrates. Silver pompano is one of the suitable species for brackish water and marine water aquaculture due to its fast growth, adaptability to different salinity regimes, good quality meat, and high market demand. Silver pompano has high Omega-3 fatty acids such as EPA and DHA in their meat (Jayakumar *et al.*, 2019). The availability of silver pompano in commercial fishery is relatively scarce and its high market demand raises the culture potential all over the Indian coast. The aquaculture of Pompano has been established successfully in many countries. Silver pompano aquaculture was done in Indo-Pacific countries, especially in China, Vietnam, Malaysia, India, and the Philippines. Total global aquaculture production of all species of pompano is more than 110,000 tonnes and appears to be growing. Silver pompano produced in Indonesia is being sold to restaurants and higher-end grocery stores in the USA. In addition to cage farming, the excellent growth rate in low-saline ponds indicates potential commercial expansion. The farming of silver pompano has been carried out in ponds, tanks, and floating sea cages (R. Jayakumar, *et al.*, 2014).

Silver pompano brooder

In India, the Central Marine Fisheries Research Institute (CMFRI) initiated aquaculture research on pompano at its Mandapam Regional Centre in 2007, and the first successful broodstock development, induced breeding, and seed production was achieved in July 2011 (Gopakumar, *et al.*, 2012).

Broodstock collection, transportation and quarantine

Wild collection and transportation of adult/sub-adults of marine finfish have a pivotal role in developing the broodstock in captive conditions. Physiological stress and physical injury of a brood fish while transportation and handling may have a detrimental effect on spawning success than any other factor (Rottman *et al.*, 1991). All the silver pompano (*Trachinotus blochii*) broodfishes are collected by wild collection from sea only. Wild collection was done from Alappuzha, Vizhinjam, Kanyakumari, Tuticorin and Ramanathapuram areas along the southwest and southeast coast of India.

Availability of the broodfishes from a particular area can be assessed by a short-term market survey or by

interviewing fishermen who target the particular fish. Experienced fishermen confirmed the presence of sub-adults of silver pompano around 200 meters from the high tide line during the months of August-December. Wild collection of fish is mainly done using Gill nets and Hook and hook-and-line fishing.

Gillnetting

Both stationary and drift gill nets can be used to collect the broodfishes. They allow a large area of water to be fished to determine migratory routes and areas of brood fish concentration. The mesh size of the net depends upon the size of the target brood fish. The nets must be checked every 15 to 30 minutes to reduce the mortality and physical stress of the fish (Rottman *et al.*, 1991; Satterfield and Flickinger, 1996).

Silver pompano fishes removing from the gill net operated off Alappuzha coast, Kerala, India

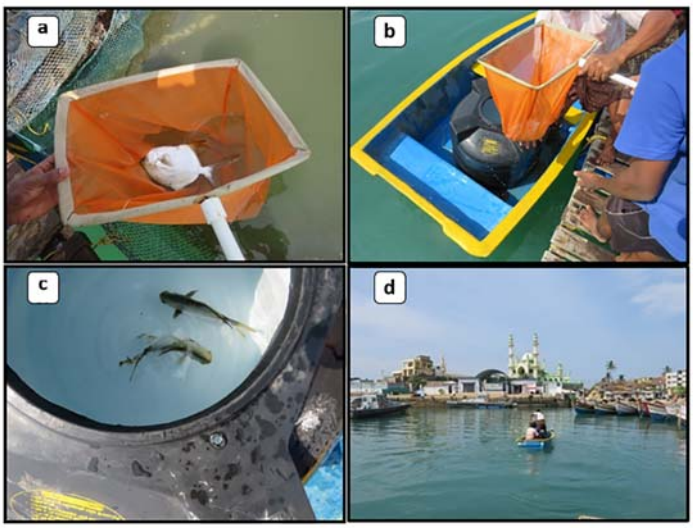
Hook and line

Sub-adults or adults of fish can be often captured by hook and line in some areas where trawling-like practices are not possible. Non-crawlable fishing grounds of coastal waters with coral or rocky outcrops which inhabit a larger variety of fish are much more suitable for hook-and-line fishing. Some fishes can be caught by hook and line only and it is a selective fishing gear which completely cuts off the by-catch quantity.

The chance for physical injury is high in this method as compared with the Gill netting. Since the fish might be under high stress during the collection process, it is critical to minimize the handling time. When the targeted fish is captured in the hook and line, immediately it has to be removed from the hook and transferred to the oxygenated water in a tank. Minimize the number of times the fish are lifted from the water, and work as quickly as possible when transferring fish to a holding tank. If there is any minor wound due to hooking, it can be treated by using Betadine ointment during the quarantine treatment.

Handling

Handling of brood fishes during the collection process should be minimal to prevent physical injury and physiological stress. Damage to the slime (mucus) layer, scales, and skin of the fish can result in infection which leads to secondary infection and may lead to mortality after the transportation. Knitted fine-mesh dip nets are recommended for handling fish to minimize injury and scale loss (Rottman *et al.*, 1991).



(a) Handling using fine mesh net, (b) Transferring to holding tank, (c) Fishes inside the tank, (d) Holding tank with fish transporting to the vehicle

Transportation

Ideally, transporting tanks/packets should be filled with water from where the broodfish are collected. Silver pompano can be transported both in the tank method and plastic bag method. The size of the plastic bag is crucial

A plastic bag with brood fish loaded onto the vehicle

for transportation and the plastic bags should be filled with 3 parts oxygen to 1 part of water. If the fish weight is more than 200 gm, it is better to opt tank method for transportation. To maintain the water temperature levels in a controlled manner, it is better to keep the plastic bag with fish inside the insulated or air-conditioned chamber in the vehicle. The collection process of broodfish always leads to a high level of physiological stress in them. To resolve this, a high level of dissolved oxygen is necessary for them to recover from the oxygen debt situation. In the tank method of transportation, one holding tank of Fibre glass or triple layered plastic tanks (Aquatech, India) of suitable capacity like 250, 500 or 1000 l (according to the quantity needed to transport and capacity of the vehicle), Oxygen cylinder and a small air pump (battery operated) can be used to transport the fish.

Tanks should be aerated. The tank should be equipped with pure oxygen regulated to maintain dissolved oxygen concentrations at a minimum of 7 ppm (Kohler, 1997). A portable generator of 220-240 Voltage can be used as a stand-by equipment to avoid critical conditions like air cut, etc. Warm water also reduces available oxygen and increases the metabolic rate of the fish, adding further physiological stress. The stocking density of fish is a critical factor which determines the survival of the brood fishes. A stocking density of 100 g/50 L is found advisable for the transportation of silver pompano. It is advisable to transport the fish during the early morning hours or night hours to avoid the temperature hike from sunlight. Ice blocks wrapped in plastic cover may be added to the holding tank during transportation to prevent an increase in water temperature.

(a)Transporting vehicle with two holding tanks, oxygen cylinder and air pump, (b) unloading the fish to the quarantine section in the hatchery.

Quarantine

After unloading the fish from the transporting vehicle, immediately transfer to the quarantine section. Quarantine tanks (fibre reinforced plastic) are of 3000 L capacity with 24 hrs. aeration facility. Stocking density inside the quarantine tanks is of crucial factor for the survival of wild fish transported to the hatchery. A stocking density of 100 gm/200 L with 200% water exchange was found optimum for the Silver Pompano quarantine facility. Various treatment procedures can be used to limit the entry of external parasites in the hatchery system from the wild-caught fishes transported. The infected fishes were primarily

treated with formalin at the rate of 10 ppm as bath treatment for 5 consecutive days which was found to be effective during the course of infection with 200 % water exchange (100 % two times) every day. Dip treatment with chloroquine phosphate (Lariago 250 mg tablets; Ipca Laboratories Pvt. Ltd.) at a dosage of 500 mg/100 L of water (Ramesh Kumar *et al.*, 2015) was found effective for silver pompano fishes. Feeding during the treatment should be avoided in the daytime to maintain the water quality parameters in a stable condition. Short-time exposure of brooders (maximum 5 minutes) in freshwater and treatment with 100 ppm formalin for 2- 5 min, were also found helpful in removing the external parasites. Quarantine treatment lasts for a minimum of 10 days (Anil *et al.*, 2019).

Wild-caught silver pompano fishes under quarantine treatment

Once the quarantine period is over, they can be transferred to the maturation tanks. It is advisable to do freshwater dip treatment quarterly for the silver pompano brooders.

Broodstock management of silver pompano in RAS

Proper care should be given to broodstock in captivity (here RAS) to get successful spawning. The viability of the larvae is very much dependent on broodstock nutrition. The nutritional components in the diet, the feed intake rate or the feeding period can all affect spawning, egg and larval quality (Gopakumar *et al.*, 2012). Improper care may lead to disease outbreaks and mortality in maturation tanks. In the case of maturation of wild-caught silver pompano in RAS, the male and female broodstock of above 1.5/ Kg is an ideal size for successful captive breeding and hatchery production of good quality larvae. Stocked fishes were fed once daily at the rate of 5 % of their body weight with fresh cleaned sardines, and anchovies. Fresh squid was given every day along with vitamin C and vitamin E tablets once a week. (Anil *et al.*, 2019).

Maturation tanks

After the fish are transferred to the maturation tanks with the recirculation facility, they are usually segregated by sex and kept in a sex ratio of 2:1 (male: female). Segregation of fishes has done by cannulation and tagging process. It is preferable to place round tanks as maturation tanks to ensure proper water circulation. Silver pompano is comparatively fast swimming fishes and any small disturbance may tend the fish to jump out of the tank and thereby tank should be covered with a net. The stocking density of broodstock maintained in RAS was 1 kg/1000 l. Pompano broodstock performed better in circular tanks with 10 t capacity. Excess broodstock can be maintained in coastal/open sea cages.

Brooder fishes in maturation tanks

Water quality requirements

Seawater to be used in the facility should enter a storage tank where it should be treated with hypochlorite solution. Water quality

requirements in the maturation tank system are a temperature of 27–29 ºC, salinity of 27–32 ppt, ammonia of d" 0.02 and a pH of 7.8–8.5, permitting adequate feeding of the broodstock while maintaining optimal and stable water quality**.**

Disease control

Adult pompanos captured from the wild or culture ponds are often infested with ciliated protozoans and/ or monogenetic trematodes. Fungal infections or injuries sustained during collection and hauling are also common. Treatments of formalin and Chloroquine phosphate at standard concentration (Kohler, 1997; Ramesh Kumar *et al.*, 2015) are usually sufficient to treat these diseases.

Feeding

Wild-caught pompano brooders can be trained to accept and maintain on trash fish, squid meat and formulated feed for over four years. Newly collected pompano should not be fed until all treatments are completed. This delay assures the fish will be hungry when feed is first offered, and prevents habituating fish from ignoring feed by presenting it to them when stress or disease agents impede their appetites (Kohler, 1997). Initially, fishes are fed with squid and trash fish by hand feeding. After a period of one month, mix squid meat (better to keep the formulated feed inside the end portion of squid) with formulated feed and train them to accept it.

Squid ready to feed broodstock

Feeding the Silver Pompano broodstock

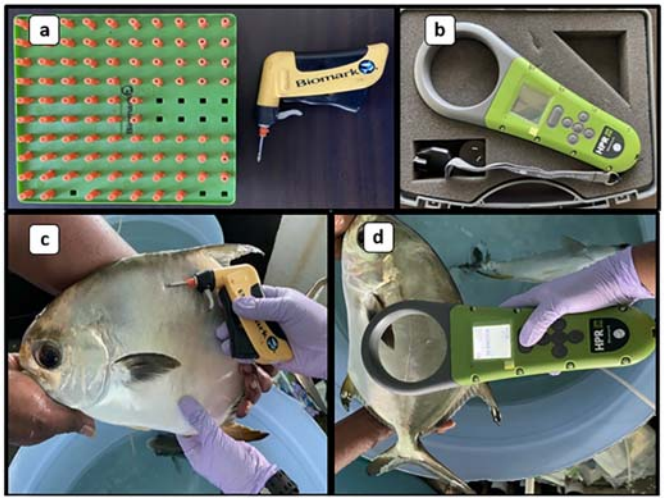
Feeding schedule maintained in Silver Pompano brood bank in CMFRI, Vizhinjam Regional Centre.

Day of the week	Feed given
Sunday	Squid with pellets
Monday	Squid and fish
Tuesday	Squid with Vitamin C
Wednesday	Squid and fish
Thursday	Squid with Vitamin E
Friday	Squid with pellets
Saturday	Squid and fish

It is very important to observe the broodfishes during the feeding so that we can understand the feeding pattern and health profile of the broodstock. If there is any reluctance to feed, the fish should be shifted to the quarantine section as immediately as possible and start the treatment.

Tagging

A Passive Integrated Transponder (PIT) tag, also known as a radio frequency device permanently marks fish internally. The tag is designed to last throughout the life of the fish providing a reliable, long-term identification method. Passive Integrated Transponder (PIT) tags act as a lifetime barcode for an individual animal, analogous to a Social Security number and, provided they can be scanned, are as reliable as a fingerprint (Gibbons & Andrews, 2004). In maturation tanks, PIT tagging can be used to answer questions regarding growth rates and maturation stages.



(a) PIT tags and tagging gun, (b) PIT tag reader, (c) PIT tagging of Pompano brooder fish, (d) Reading the PIT tag in pompano brooder fish

Normally, intramuscular insertion is preferential (below the muscular region of the dorsal fin) for pompano brooders. In normal cases, pit gag causes no mortality and few incidents of infection. The maturation stages of the fish can be assessed by cannulation of the particular fish and can be recorded in the register of the particular fish. Each tag represents a specific number which can be recorded in a register along with the growth measurements (and maturity stages) of the fish. After a definite period, fish can be recaptured and pit tag reading can be used to identify the particular fish. Various studies confirmed that pit tagging won't cause any serious impact on the survival or growth of the fish in hatchery conditions.

Ovarian biopsy to assess gonadal maturity (Cannulation)

Monitoring of oocyte development in fish was carried out at frequent intervals to determine its maturity. Oocytes collected via cannulation were observed under microscopes to analyze the diameter of the eggs. The procedure of Ovarian biopsy is given below

1. Female brooders have to be transferred to a small tank containing anaesthesia, the anaesthetic agent used is 2-phenoxy ethanol (standardized to 10mL for 100 L seawater).
2. Flexible, sterile catheters, an infant feeding tube with a total length of 520 mm and size 2.00 mm, (FG -06) are introduced through the genital pore into the oviduct for a few cm up to the ovary. A mild pressure will be applied to suck out the oocytes till a small sample of oocytes is up into the catheter and placed the sample on a glass slide.
3. After sampling, release the animal into the tank, where recovery from sedation will take place. The fish will be recovered from the sedation within 2-3 minutes. Take care to hold the fish in the tank till it recovers from sedation to avoid bleeding in the fish due to a slap at the tank due to anaesthesia
4. The biopsy sample is then examined under the microscope, measure the diameter of the oocytes, and analyze the different development stages of gonad development.

The different maturity stages are;

a) Early developing stage- Immature ovaries are relatively small with a size of 80-120 µm in diameter. Ovarian tissue was dominated by previtellogenic oocytes and strands of stromal tissue. Ovary with different stages of previtellogenic oocytes visible in the early developing stage ie; pre perinuclear

oocyte, early perinuclear oocyte, and late perinuclear oocyte.

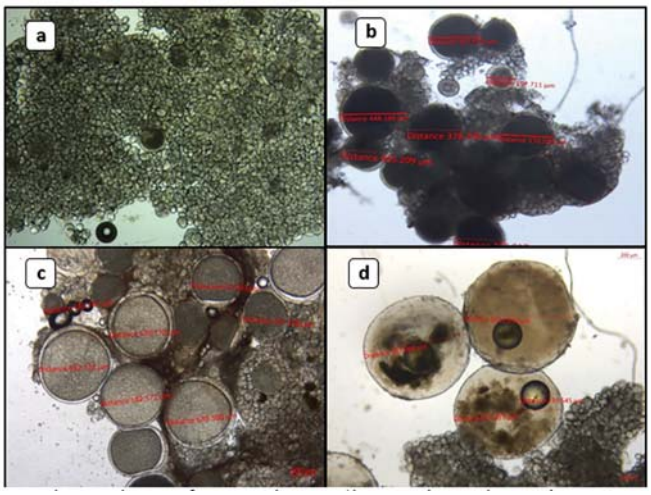
b) Maturing stage-Vitellogenesis begins with the appearance of oil droplets, yolk vesicles, and cortical alveoli vesicles surrounding the nucleus stage. Oocytes increase in size with a diameter of 350- 450 µm.

c) Mature egg stage:- The wall becomes thin due to the expansion of the ovary as it ripens and oocytes mature. Induce breeding is carried out at this stage with an egg diameter of >500 µm.

d) Spent/Atresia stage- Characterized by the presence of previtellogenic atretic oocytes without yolk granules and vitellogenic atretic oocytes with yolk granules.

Cannulation

Microscopes connected to a system with ZEN software, a modular image-processing and analysis software for image acquisition, processing, analysis and documentation for ova diameter studies.



Developmental stages of oocytes a; immature/ honeycomb stage, b; Maturing stage, c; Mature, d; Spent/Artesia

Inducement of fish using hormones

Most of the broodstocks collected from the wild maintained in the RAS do not breed in captivity. However volitional spawning of *Lethrinus lentjan* in RAS was reported from the Vizhinjam centre by Anil *et al.,* 2019. In captive conditions, there is an extreme lack of certain environmental parameters like photoperiods, rain, temperature, and water current to activate the pituitary and gonads to release hormones. However, the conditions provided under captivity need not satisfy the fishes as in the wild and may cause insufficient release of gonadotropins in captivity. In such cases, fish were induced with hormones to activate maturity and gonadal development. Hormonal inducement provides pure spawn of certain fishes under cultivation, assures timely supply of seeds and fulfils any quantity of seed demand at any time.

Silver pompano fishes are induced to spawn; when the oocyte has attained the ova diameter of the range 520- 580 µm (usually >520 µm). A sex ratio of 1:3 (female: male) has been maintained in the spawning tank for induced breeding, but 1:1 and 1:2 also had registered spawning success. The brooders selected for induced spawning must be healthy and ripe, males should be more prominent in size and weight, 2 –

3 years of age is generally selected, and 1.5 –5 Kg body weight is preferable. A minimum of 2 Kg for males and 1.5 Kg for females are a must for inducement. Spawning can be obtained either naturally or by inducing with hormones. Induced breeding is commonly practised in most commercial hatcheries for Silver Pompano. The hormonal inducement is intended to trigger the final phase (vigorous egg hydration and its release) of oocyte maturation. Before hormonal inducement, make sure that the oocytes have attained the late vitellogenic stage of development; otherwise, it doesn't work. The oocytes are matured enough to get it spawned while inducing with human chorionic gonadotropin hCG. Once the ovarian biopsy results have shown matured oocytes which completes the late vitellogenic phase, the brood fishes will be induced within 2-3 days to get the proper spawning and high fecundity. The (hCG) is a hormone widely used to induce optimal spawning of fishes. After many experimental trials, a dosage of hCG was standardized for Silver Pompano at the rate of 350 IU per Kg body weight for both males and females and administered as a single dose on the dorsal muscles. hCG can be successfully replaced by an analogue of luteinizing hormone-releasing hormone [LH-RHa des-Gly10 (D-Ala6) LH-RH ethyl amide, acetate salt]. However, the low dosage of LHRHa works well for the inducement of fish. LHRHa has super active biological properties to induce gamete maturation (ovulation and spermiation) and spawning in fishes; its low species specificity makes it worthy in the induced spawning success of a variety of freshwater and marine fishes.

Initially, a dosage of 100 µL (non-research-grade LHRHa) per kg body weight for female silver pompano and 50 µL per Kg body weight for males were administered into the dorsal body muscles of fish as a single dose or split dose. Spawning success was not achieved at this rate of hormone dosage even though the fish was mature enough. Therefore we slightly increased the dosage, and it was standardized at 150 µL per kg body for females and 75 µL per Kg body for males. Injection given at split dose works well with our system, and a photograph showing the hormonal administration is shown below. Brood fishes that responded to the above dosage continue to spawn throughout the year every 18 days. The procedure includes;

a) Selection of brood pair (1 female:2 male or 1 female:3 male). Here usually the sex ratio of 1:2.

b) Cannulate the selected female fish one or two days before inducement; male brooders need not be cannulated before inducement.

c) The cannulated oocyte diameter must be above 500- 520 µm. In the microscopic screen, a minimum of 10-15 eggs are of the above diameter to be observed to proceed with inducement.

d) If the ova diameter readings are above 500-520 µm of the cannulated female fish, then induce the fish with hormone on the following day.

e) Take the fish from the broodstock tank, anaesthetize them with 2-phenoxy ethanol and induce them with LHRHa. (either single dose or double dose). This dose will be administered on the dorsal muscles during the morning hours (9.00 am – 11.00 am).

f) After inducement, the brooders should be transferred to the spawning unit inside the hatchery in a specifically dedicated area to avoid disturbance to the brooders and the possible risk of contamination.

g) If the hormone is administrated in split dosage, then the same procedure will be carried out on the next day at the same time to introduce the remaining dose of hormone.

h) It is preferable to use circular tanks (5-ton tanks) with at least 1.2 m depth to ease the movement of brooders inside the tank. Here we experimented with the spawning in brooder tank RAS and registered 100% success in spawning. If we are keeping the induced fishes in the same broodstock tanks with

RAS, the system must be switched off at around 04.00 pm on the second day and attain spawning on the same day night between 09.00 pm to 02.00 am.

Hormonal inducement of Silver pompano

Usually, the spawning could be noted within 36 -48 hours after hormonal induction. The spawning in Pompano usually takes place between late night and early morning hours. The number of eggs spawned by pompano brooders ranged from 1.5 to 3.0 lakh eggs (female brooder, bodyweight- 2-2.5Kg). The fertilized eggs of pompano float and are scooped gently using a 500 µm net. To minimize the presence of poor-quality eggs, which usually float deeper in the water, it is advisable to collect only the eggs which float on the water surface. The post spawning mortality of fish was negligible when the fish was treated with LHRHa.

Egg collection

Collected Pompano eggs

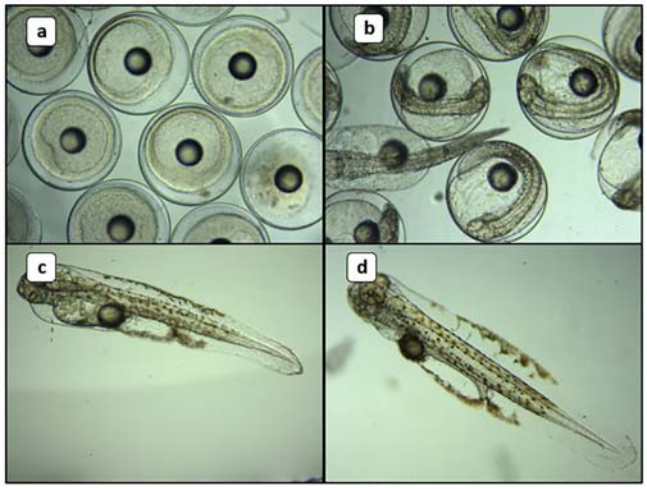
Eggs are collected using a scoop net after switching off the aeration and allowing the unfertilized/ dead eggs to settle at the bottom of the tank. A thicker layer of eggs may reduce oxygen supply to the eggs, leading to possible anoxia after a short time. Then in the temporary container, eggs must be thoroughly examined to assess their quality, number, and developmental stages. With a pipette, eggs should be taken from the floating egg layer in the temporary container and placed on a watch glass or a Petri dish for observation under the microscope. A few eggs, set under a microscope or a transmitted-light stereomicroscope, have to be observed for the egg developmental stages. As fertilized pompano eggs float in the seawater, they can be collected using egg collectors. If well-dimensioned and properly placed, these devices harvest only the floating eggs, while the dead or unfertilized ones sink to the bottom. The presence of eggs in the collectors should be checked frequently to avoid the risk of clogging the collectors leading to mechanical stress to the eggs.

Larval Rearing

Before being introduced into larviculture tanks, it is essential to evaluate the viability and condition of newly hatched larvae. A thorough examination under the microscope is required for a sample size of at least 10 to 20 fish larvae, focusing on (1) shape and dimensions (2) the presence of deformities, erosions, and abnormalities (3) Visibility of internal organs (4) pigmentation (5) Absence of external parasites. As soon as larvae hatch in larval rearing tanks, they must be distributed at a minimum stocking density of five to ten larvae per litre. To prevent mechanical stress or damage, care should be taken. A hatchling's mouth is closed shortly after hatching, and the digestive tract has not yet been fully developed. The larvae survive during this period by drawing on the reserves contained in the yolk sac.

Larvae stocked at a density of 40000 per 5000 L FRP tank filled with 4000 L filtered seawater (stocking

density of 10 nos/L). The tanks should be aerated mildly and contain green water with a cell density of 1 x 10 / mL. The larvae's mouth opens at 3dph and measures around 230 mm. Larviculture tanks should fed enriched rotifers at a density of 5-8 nos. per mL from 3 dph to 14 dph. Rotifers must be co-fed with enriched Artemia nauplii during 8-12 dph, and thereafter maintain at a density of 3-5 nos./mL with enriched Artemia nauplii alone up to 18 dph. Co-feeding with Artemia should be continued until 18 days after weaning to larval inert feeds. It is possible to feed exclusively on larval inert feeds from 19 dph onward. From 8 to 14 dph, it can be progressively raised from 10% to 100%. The starting point for nursery raising might be 25 to 30 dph. Currently, an artificial feed with a size of 800µm might be offered. After that, fingerlings were given floating extruded larval meals with a gradually larger size range.



(a) Fertilized eggs, (b)Hatching started, (c) 1 dph, (d) 2 dph

A 100% water exchange every day is recommended. Throughout the whole larviculture phase, water quality parameters like as salinity, temperature, pH, oxygen content, and ammonia should be continuously monitored. Fingerlings that range in size from 1 to 1.5 inches can be given to farmers for stocking in marine or brackish water cages for additional nursery raising and grow-out farming.

Summary

The control of the reproductive function of marine fishes in captive conditions is essential for the sustainability of commercial mariculture and brackish water culture production. The induced breeding of marine fishes can be eased by controlling the reproductive hormones of the brain, pituitary, and gonad. The Recirculatory Aquaculture System for keeping the brooders, the process of PIT tagging for easy identification of fishes for cannulation and ovarian biopsy studies, broodstock maintenance with adequate feed, and frequent monitoring of oocyte development eased the gonadal development in the case of Silver pompano. The female fish attained maturity at a minimum weight of 1.4 Kg. We have never faced stress in fish during anaesthesia, cannulation, and hormone inducement. The hormone dosage standardization only took a bit more time in the entire process of Silver pompano breeding. Single-dose doesn't fit in the initial phase of the breeding programme, where we administered the hormone in split doses. Later the same fish with a lower dosage at a single dose acts perfectly and attains spawning success with less response time. One of the brood pairs starts laying eggs volitionally after three years of induced spawning. The continuous effort for standardizing the breeding of Silver pompano at Vizhinjam Regional Centre of CMFRI under financial assistance from NFDB aids in creating a national brood bank for Silver pompano at the centre.

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