Original Research Article

A MOLECULAR STUDY ON THE EXPRESSION PROFILE OF STRESS-RESPONSIVE GENE/S EXPRESSION IN FISH SPECIES AT KOLLERU LAKE ANDHRA PRADESH; A COMPARATIVE ANALYSIS ON FISH DIVERSITY AND POPULATION

Abstract

Kolleru Lake in Andhra Pradesh is one of the most significant biodiversity hot spots in India. The Lake is 6,121sq km long, with a flow of 65,000 to 1.1 lakh cusecs of water into the Lake. It offers habitat for a wide range of animal life, including wild animals, birds, and fishes. Over the recent past, a drastic decrease in fish population in the Lake is being reported, that not only adversely impacts biodiversity but also affects the food chain as well. Several risk factors involved in the reduced biodiversity of fish in the lake are under constant evaluation by researchers. Among these, some of the major ones identified are illegal fishing, a rise in water pollution, alien species of fishes and other aquatic animals. The growing concern, for now, is how to restore fish population and biodiversity. Scientists are working on a molecular level to understand water pollution and lack of nutrients on fish growth and development. Stress is a crucial physiological state which all living organisms face, and respondon either unique or a common modality. Several stress-responsive genes expressed during the unfavorable conditions, allow coping up with the drastic change in the surroundings. The expression of stress-responsive gene(s) also depends on environmental factors/triggers. The survival of fish in unfavorable conditions largely relies on how effective animals respond against the changing environment. We aimed to investigate significant risk factors for the reduced fish population in Kolleru Lake in Andhra Pradesh and the corresponding stress responsive genes.

Keywords: Kolleru Lake, Fish Diversity, Stressors**,** Stress Responsive Genes, Environmental Risk Factors.

Introduction

One of the largest fresh water wetlands of India, believed to have initially extended over 1050 km^2 and existed as a coastal lagoon during the mid-Holocene period in the Bay of Bengal, gradually turned into a fresh water lake as several rivulets continued to drain into the lake. This lake, called the Kolleru lake, is located in the Krishna district of Andhra Pradesh (16.65^{0}N, 81.22^{0}E) along the east coast of India and serves as a vital flood-balancing reservoir between the Krishna and Godavari delta plains (Naga Kumar et al., 2016). Several ecosystem and societal services like flood mitigation, food security, landscape aesthetics and biodiversity resources are offered by the wetlands, and therefore are the most threatened habitats with direct encroachments for human benefits like agriculture, aquaculture, livestock farming, biological resource use, exposure to invasive use, urbanization and climate change. A global reduction of wetlands by 64.71% was reported by Davidson (2014) and that the rate of loss was high in Asia. In aquatic ecosystems, fish are the important food produce that satisfy the needs of nearly 4.5 billion people in terms of dietary protein ranging from 15 to 20% of per capita intake (greenfacts.org/en/fisheries). Of this, freshwater fish make up more than 6% of the world's annual animal protein supplies (FAO, 2018) for humans and in India, it is 12.8%. Interestingly, 2/3rd of India's fish supply is estimated to come from fish farms (cultures) rather than natural fresh water and marine bodies, by 2030. The top five states in India producing inland fish are Andhra Pradesh (28.45 metric tons), West Bengal (15.57 metric tons), Uttar Pradesh (6.29 metric tons), Kerala (5.35 metric tons) and Bihar (5.88 metric tons). In Andhra Pradesh, the high fish producing districts are Godavari, Krishna and Nellore. The Kolleru lake is a haven for migratory and resident birds viz. Siberian cranes, ibises, painted storks, grey pelicans, water fowls etc. and was demarcated as Kolleru wild life sanctuary (Nageswara Rao et al., 2004;2010).

Current Fish Biodiversity in Kolleru Lake

The popular fresh water species of Indian rivers are *Labeorohita*, *Hilsailisha*, *Pulasa*, *Catla*, *Tilapia* (Cichlid), *Mystustengara* (Tengra), *Magur* (Walking cat fish), *Ailiacoila* (Kajuli), Rani (Pink Perch), Ritha (cat fish) (thinkpedia.in/species-of-fishes-in-India).A study on ichthyofaunal diversity of kolleru lake recorded 78 fish species belonging to 14 orders, 37 families and 57 genera. Of these, order cypriniformes was dominant with 26 species contributing to 41.94% of the total species followed by Perciformes – 22.58%, Siluriformes – 19.36%, Channiformes 6.45%, Anguilliformes – 3.23% and Osteoglossiformes – 1.61% at kolleru lake. Of the different orders of the pisces available at kolleru lake, Perciformes was found to be dominant with 7 families, genera and species that contributed to 43.75%, followed by Mugilliformes contributing 3 families (8.75%), Osteoglossiformes contributing 2 families (12.5%), Cyprinodontiformes, Siluriformes, Gonorhynchiformes and Pluronectiformes, each contributing 1 family (6.25%) at upputeru creek. Despite the potential as a rich fish resource, Kolleru lake is under anthropogenic threats as well as seriously affected by ecological problems like weed infestation, siltation, pollution, eutrophication and agricultural encroachments (Barman, 2004; Bharatha Lakshmi et al., 2015; Krishna et al., 2016).

Stress and Stressors

Stress, often defined as the non-specific result of any demand upon the body (Selye, 1978; Schreck and Tort, 2016) reflects homeostasis including all the hierarchical levels in a networked biological system. The stress responses are therefore heterogeneous and the stressors vary widely in terms of type, individualized sources and effects. The stress response patterns in fish depend on several factors including the stressful experiences in early life, the vertical transmission of stress-prone phenotypes, the degree of individual phenotype plasticity, the robustness and variety of epigenetic network related to environmentally induced changes, and the intrinsic behavioral responses of each individual organism. The stress phenotypes are deciphered by the simplistic analysis of both proximate and evolutionary causes. The ’stressotope’, an analytical frame work is determined by the common environmental selective pressures that elicit common multilevel acute stress induced responses and produce a measurable allostatic load in the organism. Upon exposure to stress, there could be subtle to profound variations in cell states, molecular mediators and systemic networks that could be acquired or inherited.

General stress predictions

Common predictors of stress related responses should be ascertained to the accurate selection of more explanatory variables. For instance, a physical variable like temperature may act as a stressor both at high as well as low levels relative to the normal physiological temperature limits of the species. It enables understanding of the adaptive pathway of each species with a more realistic description of intra- and inter-species specific ‘natural’ vs. ‘distressed’ pathways of stress responses. Another important consideration while assessing the stress responses is the inclusion of variations in the maturation of primary and secondary immune organs rather than mere size variations within the populations. Similarly, the resilience of low abundance pathogen communities in a given environment will have a substantial impact on the species’ behaviour to the stresses (Lynch and Neufeld, 2015). Thus, an analysis of the stress-related phenotype describes physiological outcome of specific gene networks at ontogenetic and phylogenetic levels (Balasch and Tort, 2019). ‘Biocenosis’ refers to the biotic components while the term ‘Biotope’ refers to the geological environment.

Common stress responses

Flik et al., 2018, The function of the stress response is to accommodate appropriate modifications in the energy budget, immune system and hormonal make up of the organisms to withstand the environmental stresses (Flik et al., 2018). The concept of stress refers to the idea of an altered status and physiological exceptionality. It is essential to note that, despite the diversity, all living species demonstrate a common mechanism and a conserved response in adapting to the stressors, the stress course and the response of the organism. Responses to the stress are key to maintain the physiological, cellular and molecular stability, in toto, the homeostasis of the organism. Among the several mechanisms that can be chosen to maintain homeostasis, the determining factors include the species itself, the environmental conditions and the stressor attributes like intensity, duration and predictability. The stress responses emerge when the stimulatory demand exceeds the natural regulatory capacity of the organism (Koolhass et al., 2011).

Review of Literature:

FAO (2018). [1] The Kolleru Wildlife Sanctuary in Andhra Pradesh known for its rich bio-variety is under danger from unlawful fishing tanks, modern contamination and various different variables. Kolleru, one of the biggest freshwater lakes in India, (it was assigned an asylum in October 1999) is arranged between the Krishna and West Godavari regions of the state. Kolleru's water accessibility relies upon various sources. Budameru, Tammileru, Ramileru, Gaderu and Bulusuvagu waterways just as various streams channel their waters into the lake. The catchment area of Kolleru is around 6,121sq km with a progression of 65,000 to 1.1 lakh cusecs of water into the lake. The lake channels out into the Bay of Bengal through the Upputeru channel, it’s just outlet. Kolleru is quite possibly the main wetlands of Indium. The lake upholds 200 types of

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[1]. FAO (2018). The State of World Fisheries and Aquaculture 2018 – Meeting the Sustainable Development Goals. The State of World Fisheries and Aquaculture. Food and Agriculture Organization of the United Nations, Rome.

birds, including the basically jeopardized Spoon-charged Sandpiper. The lake fills in as a searching ground for occupant just as transitory birds. As of now, 6,000 Spot-charged Pelicans, 5,000 Painted Storks and 5,000 Asian Open bills are assessed to be found in Kolleru. The lake additionally upholds 63 types of fish. The Kolleru Lake assumes a fundamental part in retaining carbon, which thusly, mitigates the impacts of an unnatural weather change. At present, the emanation of carbon into the air is a significant worldwide issue. This freshwater lake goes about as a carbon sink like some other thick woodland region. It likewise goes about as a channel for toxins delivered because of modern action.

Balasch Joa.n Carles and Tort Lluís (2019)[2], broadly, the stress responses in fish are categorized as primary, secondary and tertiary responses (Barton, 2002). Primary response is associated with instantaneous release of catecholamines viz. adrenaline and nor-adrenaline from autonomic nervous system, hypothalamus-pituitary gland-interrenal gland activation, release of corticotropin-releasing factor (CRF) from the pre-optic area of hypothalamus, activation of pituitary corticotrophs and release of adrenocorticotrophic hormone (ACTH) in the blood. The release of cortisol, a stress hormone, is induced by the ACTH of the interrenal gland (Bonga, 1997) and this hormone helps in highly adaptive stress response of the organism and reflects its stimulated learning and neural plasticity (Sorensen et al., 2013). The secondary responses, which are temporary, span a cascade of metabolic and physiological changes manifested as modified blood glucose and lactate levels (Barton and Iwana, 1991) and expression of several heat shock proteins like Hsp 70 (Waliaand Balkhia, 2016; Eissa and Wang, 2016). If the challenges continue to exist, then the organism exhibits tertiary level response that comprises altered swimming behaviour, appetite, reproduction (Barton, 2002), muscle wasting, immunosuppression and attenuated microbial resistance (Huntingford et al., 2006).

Objectives of the Study:

The study aimed with the following objectives-

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[2]. Balasch Joa.n Carles and Tort Lluís (2019). Netting the Stress Responses in Fish. Frontiers in Endocrinology, 10, 62. doi: 10.3389/fendo.2019.00062

1. Identification and profiling of risk factors associated with decreased fish population and biodiversity in Kolleru Lake in Andhra Pradesh.
2. Identification of gene/s involved in stress state in fishes, how does these gene/s expression in liked with reduced population and biodiversity of fishes at Kolleru Lake Andhra Pradesh.
3. Real-time PCR analysis of expression of stress-responsive gene/s in fish species at Kolleru lake Andhra Pradesh.

Results & Discussions

Methodologies for Measuring Stress

While short term stress response is reasonably well understood, knowledge on chronic response is inadequate (Madaro et al., 2015) and the need to understand the molecular mechanisms of stress so as to develop appropriate detection mechanisms at an early stage and safeguard welfare of the fish is warranted (Gomati et al., 2005; Prunet et al., 2012). Earlier studies considered cortisol and Hsps as potential stress markers (Barton, 2002; Basu et al., 2002) but they could not stand the marker validation tests by subsequent researchers (Iwama et al., 2004; Martinez-Porchas et al., 2009). With the advent of novel and powerful technologies in recent times, RNA and protein expression profiling of tissue and / or whole body samples could be correlated to secondary and tertiary stress responses of the organisms (Feder and Walser, 2005; Sopinka et al., 2016; Faught et al., 2016). From a typical case study on the Atlantic Salmon (Salmo salar), it has been inferred that RNA/Transcriptional assessment is the dominating approach for understanding long term stress responses (Hoem and Tveten, 2020). As experimental studies on stress responses are highly varied with the selection of different variables like physico-chemical factors, species, duration of exposure to stress etc., it is not wise to generalize or identify one or a few selected markers. Also, variations in the transcriptional levels of coding genes cannot be directly extrapolated to the proteins as all the RNAs may not get translated to active, functional proteins.

The current gaps in understanding the mechanism of stress management at organismic level could be abridged by genetic-surveillance-driven selective breeding to increase tolerance to specific stressors (Prunet et al., 2008; Eissa and Wang, 2016). Regulation of gene expression at transcriptional and post-transcriptional level is intensively studied in mammals and not so in other animal species. However, time dependent 'on and off' regulation of mRNAs is observed from different studies (Gotting and Nikinmaa, 2017; Tadiso et al., 2011; Mollet et al., 2008). It has been suggested in fish that mRNA stability plays a significant role in hormone mediated stress responses. Inhibition of general protein translational machinery and upregulation of proteins involved in hormonal mediated stress responses in fish has been reported (Gotting and Nikinmaa, 2017).

Stress, related to a particular biotope, is commonly measured in terms of glucocorticoids and / or varied behavioural patterns upon exposure to stressors like predation risk, parasite load, metabolic trade-offs associated to seasonal resource shortages, high density populations, artificial habitats, HIREC (Human Induced Rapid Environmental Changes) influences etc. (Gallagher et al., 2017; Laundre et al., 2014). It is proposed that complexity of the stressotope to be assessed against a minimum common number of informative variables like natural environmental insults including physical factors like temperature and oxygen, salinity (varying tolerance levels in freshwater and marine species), photoperiod, abundance of pathogens, landscape of fear (LoF), species-specific intersex differential activation of stress.

Hypoxia responsive genes in fish

Dissolved oxygen level is one important factor that greatly influences the fish survival and needs to be constantly monitored. If they DO level remains below 1-2 mg/L for a few hours, fish growth and immunity is badly affected and may even result in death due to oxygen insufficiency and / or infections (Abdel-Tawwab et al., 2019). Hypoxia affects fish behavior, performance of gills, osmo-respiratory compromise, fish performance and production, biochemistry and physiology, immunity and bacterial infections, DNA and gene expression. Transcriptional regulation of hypoxia induced genes appears to be determined by the activation of a transcription factor viz. hypoxia-inducible factor (HIF-1) which is a heterodimer composed of α and β sub-units. The numbers of HIF-1α mRNA copies were found to increase significantly in response to both acute and chronic hypoxia in European Sea bass while the number remained unchanged in fish exposed to hyperoxic conditions (Terova et al., 2009). The tolerance to hypoxia is demonstrated to be governed by multiple genes and that HIFs bind to regulatory regions of the hypoxia-inducible genes, resulting in HIF-regulated gene activation (Ortiz-

Barahona et al., 2010; Mohindra et al., 2013; 2016). Further, hypoxia induced DNA damage was observed in the liver of African cat fish, Clariasgariepinus (Al-Salahy, 2006; Mahfouz et al., 2015) but not in the white muscles. Heme oxygenase 1 is a cytoprotective enzyme involved in cellular action against hypoxia stress and occurs in 2 forms HO-1a and HO-1b. mRNA of both these proteins were upregulated significantly in the gill and liver but downregulated in the brain suggesting the divergent evolution of the genes (Guan et al., 2017; Tzaneva and Perry, 2014; Taylor et al., 2003).

Rashid et al. (2017) used LAMPP (Linux Apache MySQL PHP and Perl) technology and developed a database of hypoxia responsive genes in fish (HRGFish), with the analytical tools integrated. HRGFish is a repository of genes reported to be involved in hypoxia response in fish. Several upstream sequences, promoter regions and different sets of orthologous promoters from the genes in the database are analyzed to understand the different components of gene regulation like regulatory elements, hypoxia response element (HRE) orientation, tolerance and susceptibility of the species against hypoxia. Upstream analysis includes the computation of CG dinucleotide frequency, HRE motifs (graphical forms) and localization of CpG islands (graphical plot) in the upstream sequence of the selected gene. Amplification of the conserved exons of a gene to validate homologous genes in different species is facilitated with the primers designed for cross species PCR (Cs-PCR) and the technology provides a greater insight into the stress management in the fishes (Rashid et al., 2017). The cs-PCR amplification, a commonly employed technique for retrieving common regions of gene sequences among related species, was applied by Iyengar et al. (1998) to infer the ancestral state of human polymorphisms. The well-equipped HRG Fish resource presents opportunities for state-of-the art research related to the study of hypoxia response as well as genetic relatedness among the species and genetic improvement programs of commercially important aquaculture species. The users of the database are provided with a platform for analysis of gene sequences, design of primers, analysis of gene sequences, and upstream annotation of genes. The different tools and programs are graphically represented under the menu item ‘About database’ for the convenience of the users. As per the last update dated 29.04.2019, 48 gene types belonging to 65 species of 41 families with a coverage of 1891 genes. These were compiled from the data published in research articles.

Some important genes of mention are Ca^{2+}/Calmodulin dependent protein kinase, Ceruloplasmin, connective tissue growth factor, CREB binding protein & regulated transcription co-activator, erythropoietin, ferritin, fibrillarin, glucose transporter 1 to 3, Heme oxygenase 1 & 2, IGF binding protein 1 to 3, myoglobin, VEGF A to C and others. For further details, readers are advised to visit the HRGFish database (mail.nbfgr.res.in/HRGFish/). Architecture and data flow diagram of HRGFish, (the database of hypoxia response genes in fish, is first of its kind to the best of our knowledge), are indicated for understanding the utilities offered by the database (Rashid et al., 2017) and that provides the workbench to the scientific community who are engaged in understanding the evolution and ecological adaptation of the fish species exposed to hypoxic conditions. Researchers adopted different approaches to identify the hypoxia responsive genes and the alterations in their expression. Studies indicated the induction of gene expression in hypoxia through nuclear transcriptional factor which is subsequently followed by the transcriptional activation of human EPO gene enhancer (Semenza and Wang, 1992).

Gene expression in the brain and kidney of rainbow trout

Identification of differentially expressed genes in rainbow trout upon exposure to environmental stressors was achieved with the implementation of microarray technologies and the study envisaged time course comparisons of stress responses in the brain and kidney, consequently recognizing a set of genes which are diagnostic for evaluation of stress responses. In this particular study (Krasner et al., 2005), fish were subjected to handling stresses and the gene expression profiles were analyzed with gene ontology categories. The major renal targets included mitochondria, extracellular matrix and endopeptidases, while temporal alterations in different proteins of the brain tissue were significant. Contrary to the metal ion binding proteins, glycolytic enzymes and motor proteins that were transiently induced, stress and immune response genes, cell proliferation & growth, signal transduction & apoptosis, protein biosynthesis and folding changed in a reciprocal fashion. In total, from the study, 48 genes that showed strong correlation of expression profiles and regulation in stress, were identified by microarray experiments. Although the number of differentially expressed genes was significantly higher in EST than in genes selected through gene ontology annotations, 63% of stress-responsive genes belonged to the group. In a nut-shell, the study reported that the stress responses differ both by 'target functions' and 'time course' in the tissues of brain and kidney. Initial transient induction of motor and metal ion binding proteins followed by slow adaptive responses was characteristic of brain while prolonged stress on kidney causes the degradation of its extracellular matrix. Some of the major differentially expressed genes in brain include signal transduction proteins like RAS &GTPase, chaperones/Hsps, metal ion binding proteins/potential-driven transporters, cytoskeletal/myofibril proteins in the brain, mitochondrial/electron transporter proteins, endo & metallopeptidases, extracellular & Zn^{2+} binding proteins were significant in the kidney (Krasnov et al., 2005).

In Atlantic Salmon fish, exposure to stress in the early developmental period viz. embryonic and psot-hatch periods was demonstrated to have late effects on the methylome, transcriptome and growth response. The study was targeted to understand the responsive alterations in the fish transcriptome during subsequent exposures to acute stress and the role of methylation in these responses. Corticotropin releasing factor 1 was strongly downregulated while the glucocorticoid receptor and 3-oxo-5-alpha steroid 4-dehydrogenase 2 gene expression was upregulated. It was noticed that the fish manifested 'switching away' from growth in the process of coping up with the stress, following acute stress exposures in EPH fish. Further, major histocompatibility 1A, T-cell receptor and toll-like receptor are found to be involved, affecting the robustness of the organism.Differential DNA methylation in the stress response genes in the regions mapping less than 500 bases is suggestive of the involvement of epigenetic mechanisms. The results of the study strongly suggest the invocation of stress treatments during early embryonic development will boost the fish productivity (Robinson et al., 2019).

Seasonal and sudden thermal shock is a common abiotic stress thrust upon fish due to their ectothermal nature. These temperature alterations in the ambient environment induce profound changes in the physiology, behavior and cellular mechanisms of the organisms (Barat et al., 2015). With the result, cellular transcriptomic (mRNA pool) changes are rapid and dynamic in fish experiencing the environmental stress (Buckley et al., 2006), although they are not diagnostic in evaluating the stress induced responses in the organism. Several significant studies on the expression level of various genes have been reported with respect to environmental and internal stressors like temperature, oxidation, apoptosis, protein folding, energy metabolism, protein synthesis, membrane fluidity and immune function (Basu et al., 2002; Buckley et al., 2006; Kassahn et al., 2007; Perez-Casanova et al., 2008; Podrabsky and Somera 2004; Yiangou et al., 1998). The major classes of genes that are triggered in stressed conditions are those of heat shock proteins, anti-oxidative enzymes and metabolic enzymes, more particularly of carbohydrate metabolism. The transcriptomic investigations of the effects of environmental stresses on fish have been reported in the last two decades (Hori et al., 2010; Lewis et al., 2010; Quinn et al., 2011; Huth and Place, 2013; Liu et al., 2014; Narum and Campbell 2015; Rebl et al., 2013; Smith et al., 2013). Deep RNA sequencing on Illumina paired-end platforms was done for characterization of the genes and molecular pathways involved in the response of snow trout (*Schizothoraxrichardsonii*) to heat shock. From the study, Barat et al. (2015) identified approximately 65 differentially expressed genes, out of which 8 were heat stress-responsive genes upregulated in liver tissue of *S. richardsonii*.

At times, non-specific responses elicited against physical, chemical and other perceived stressors are crucial for the adaptive behavior of fish to maintain homeostasis. However, in the case of heavily acute and persistent existence, the stressors themselves become maladaptive and adversely affect the health of the fish (Barton, 2002). Prolonged exposure to stress due to the nature of farming practices may culminate in reduced performance and health of fish. Several stressors including water quality, diet and management practices have been studied and are confined to transcription level analysis. Quantitative reverse transcription PCR (RT-qPCR) is the dominating technology followed by microarray assays. As correlation studies between transcript expressions and protein levels are minimum, it is imperative to conduct more studies to better understand the physiological mechanisms underlying the stress. Further, an indepth understanding of the regulatory mechanisms operating on pre- and post-translational levels that might be affected by environmental factors should be attempted.

Conclusions

Increased environmental and internal stressors are a cause of concern for the fast decline in fish biodiversity in general and more so in kollerulake because of heightened anthropogenic pressures for enhanced commercial production of selected species. Stress responses in terms of physiological changes are exhibited by all organisms, however to varying levels depending on the internal genetic make-up, intensity and duration of the stressor impacts, hormonal regulation and gene expression levels. Majority of the stress regulatory responses include the hormonal pathways that constitute significant part of the signal transduction mechanisms. In addition, different heat shock proteins, metal ion binding proteins, transporters, metallopeptidases and others are expressed in a tissue specific pattern and enable the organism to adapt to the persistent stress. However, continued decline in the species and consequent extinction are imminent if the stressors are of high intensity and duration. These challenges need to be addressed by minimal interference with the environmental factors, restoration of ecosystem original composition and reduced culture practice pressures. Also, a thorough understanding of the transcriptional, post-transcriptional evaluation of the whole body proteome and/or tissue specific proteomic studies would facilitate a holistic approach to control biodiversity loss and healthy fish productivity. One another way of sustaining biodiversity of these vulnerable wetlands is through local community participation in conservation measures and appropriate rewards to continue their involvement. Thus, a comprehensive approach in terms of scientific investigations with the utilization of advancing technology and the re-introduction of traditional aquaculture practices with the active involvement of local fishing community would be vital in a beneficial management of food resources.

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