Quantitative traits evaluation of different strains of indigenous culturable species rohu *Labeorohita*

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DOCTOROFPHILOSOPHY

By

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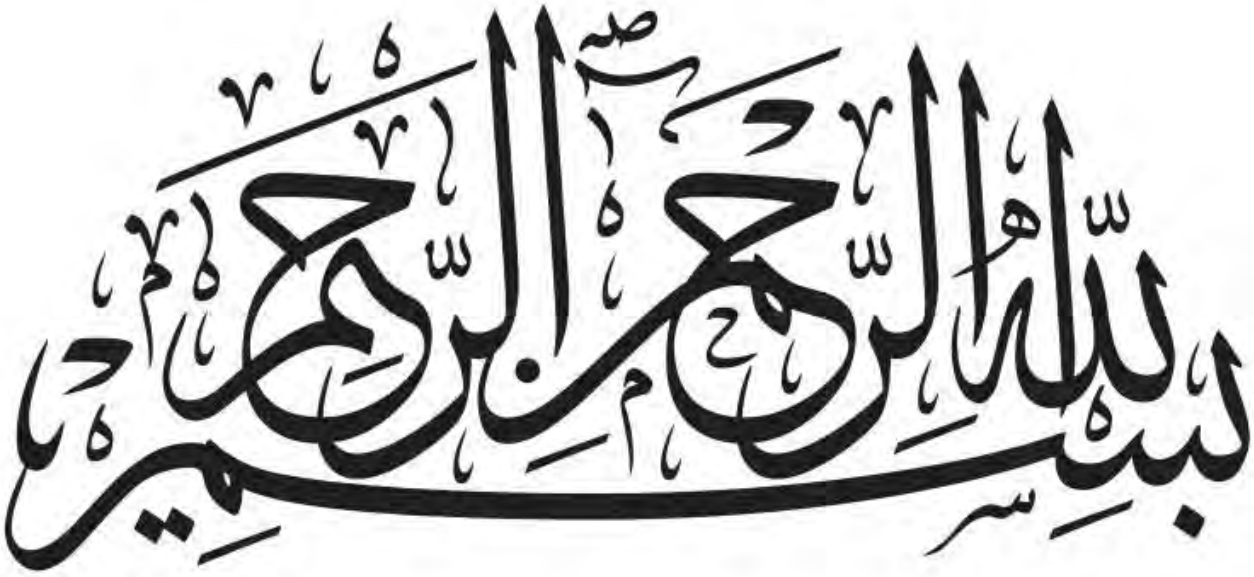

IN THE NAMEOFALLAH,THE MOSTMERCIFUL THE MOSTBENEFICENT

AND

THEMOST COMPASSIONATE

CERTIFICATE

Thisdissertation **Quantitative traits evaluation of different strains of indigenous culturable species rohu *Labeorohita*,**submittedby**Muhammad Noorullah** isaccepted in its present form by the Department of Zoology, Faculty of Biological sciences,Quaid-I-AzamUniversity,Islamabad,assatisfyingthethesisrequirementforthedegreeofDoctorof Philosophy in Fisheriesand Aquaculture.

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Date: _____

Declaration

I hereby declare that the work presented in the following thesis is myown effort, except where otherwise acknowledged, and that the thesis ismyowncomposition.Nopartofthisthesishasbeenpreviouslypresented foranyotherdegree.

MuhammadNoorullah

Dedication

I dedicate this humble effort to theHOLYPROPHETHAZRATMUHAMMAD (PeaceandBlessingofAllahBeUponHim).

The reason for the creation of this universe&

I dedicate this work to my father and my mother, who taught me the art of never giving up. Without their endless love and encouragement, I would never have been able to complete this work. I love you both and I appreciate everything that you have done for me.

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ListofAbbreviations

Abbreviations	FullNames
°C	DegreeCelsius
µg/L	Microgramperliter
µl	Microliter
ANOVA	Analysisofvariance
BLAST	Basiclocalalignmentsearchtool
BSA	Bovineserumalbumin
CMs	Charbanda Mardan Strain
COI	Cytochrome oxidase subunit I
CP	Crude protein
CTmax	Critical thermal maxima
CTmin	Critical thermal minima
DNA	Deoxyribonucleicacid
dNTPs	Deoxynucleotidetriphosphate
DO	Dissolvedoxygen
EDTA	Ethylenediaminetetraaceticacid
FARF	Fisheries and aquaculture research facility
FBW	Finalbodyweight
FCR	Feed conversion ratio
GIFT	Genetically improved farmed tilapia
HCT	Hematocrit
He	Expected heterozygosity
Ho	Observed heterozygosity
hrs	Hours
IBDs	Islamabad Strain
IBW	Initialbodyweight
IgM	Immunoglobulin
K2P	Kimura 2 parameter

ln	Naturallog
LOE	Loss of equilibrium
LSD	Leastsignificantdifference
mg/L	Milligramperliter
min	Minutes
MKs	Mianchannu Khanewal Strain
MS222	Tricainemethanesulphonate
NBT	Nitroblue tetrazolium
NKEF	Natural killer enhancing factor
PBS	Phosphatebuffersaline
PCR	Polymerase chain reaction
pH	Potentialofhydrogen
PIT	Passive integrated transponder
ppm	Partspermillion
RBC	Red blood cell
RPM	Rounds per minute
SDS	Sodium dodecyl sulfate
SGR	Specific Growth Rate
SPs	Sherabad Peshawar Strain
TMs	Tawakkal Muzaffargarh Strain
USs	Upper Sindh Strain
WG	Weight gain
WKs	Wild Kabul Strain
WRs	Wild Ravi Strain

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Abstract

Strain evaluation and selection are crucial to address the disarray in aquaculture of any country and start a genetic improvement program. Unfortunately, Pakistan is far behind in launching any strategy for the genetic improvement of the existing fish stock, thus facing many challenges in this sector, including the availability of quality fish seed, stunted growth, higher mortality, higher FCR and overall low per acre production compared to many countries. limited literature is available on the genetic structure of indigenous culturable fish species of Pakistan. Thus, the current was designed to address this issue and determine the genetic diversity among eight different populations/strains of highly culturable species *Labeorohita* and then compare their growth performance, thermal tolerance, innate immunity, and disease resistance. Among eight different stains, six captive-bred strains, USs, SPs, TMs, MKs, CMs and IBD-s were collected from Fish Hatcheries, Upper Sindh Fish Hatchery, Carp Hatchery and Training Center, Peshawar, Tawakkal Fish Hatchery Muzaffargarh, Govt. Fish Hatchery, Mianchannu, Govt. Fish Hatchery, Charbanda, Islamabad Fish Hatchery and Training Centre, respectively, while two wild-strains WKs and WRs were collected from River Kabal and River Ravi, respectively. Rivers and Fish Hatcheries across the country were selected based on their geographical location and diverse fish culture practices. Genetic diversity among different strains was determined using DNA barcoding employing cytochrome oxidase subunit I (*COI*) and three different microsatellite markers, i.e., Lr-37, Lr-29, and Lr-28. The findings revealed an elevated level of observed heterozygosity (Ho) across all strains (Avg: 0.731), though less than the expected heterozygosity (He). Moreover, TMs and WRs exhibited the highest He, while TKs and CMs demonstrated the lowest value. Overall, FIS values observed for all strains with selected markers were positive. The DNA barcoding utilizing the CO1 gene also indicated significant genetic variation among various strains, as demonstrated by the formation of clades in the phylogenetic tree separating the strains into two distinct clusters that subsequently branch into sub-clusters. Overall, TMs showed the highest heterozygosity as compared to other strains. To evaluate quantitative traits like growth rate, thermal tolerance, disease resistance, and innate immunity, advanced fry of all stains were procured and reared at the Fisheries and Aquaculture facility at Quaid-i-Azam University. The growth performance, health status, and innate immunity of advanced fry and fingerlings of all strains were conducted under controlled conditions in an indoor facility and under semi-intensive culture conditions in the earthen communal ponds, respectively. For identification, fingerlings of all strains

were PIT tags and stocked in the earthen communal ponds. The 90-day growth performance data of all strains, both at advanced fry and fingerlings stages in controlled and semi-intensive culture systems respectively, revealed the highest final weight, weight gain %, SGR %, and final biomass of TMs followed by WRs. However, CMs, SPs and IBDs showed comparatively poor growth performance. Moreover, WKs under controlled conditions while TMs under semi-intensive culture conditions showed the highest % survival, whereas IBDs under both culture systems showed the lowest % survival. The mRNA level of growth-related genes, Myogenin, MyoD and IGF-11 in liver and muscle tissues of all stains showed a similar pattern, i.e., the highest expression in TMs and the lowest in the CMs and SPs. Moreover, significantly higher values of haematological indices (RBCs, HCT %, HGB and WBCs), innate immunity parameters (Total protein, IgM, Lysozyme activity, Respiratory burst activity and Phagocytic activity) and relative expression of Natural killer enhancing factor (*NKEF*), Lysozyme C, Lysozyme G, and TNF-α also indicated the comparatively improved health status and strong immunity of TMs followed by WKs. However, CMs followed by IBDs showed comparatively low values of all haemato-immunological indices. The status of antioxidant enzymes (CAT, SOD, POD, and GST of fingerlings of all strains also showed a similar trend, i.e., the highest activity in TMs followed by WRs while the lowest activity in the gills of CMs and IBDs. The proximate composition of the fingerlings of all strains after 90 days of rearing in communal ponds also showed the highest crude protein % and the lowest moisture (%) in the TMs, followed by WRs, while USs and SPs showed the lowest value of crude protein (%). For the evaluation of critical thermal tolerance (CTmax and CTmin), fingerlings of all strains were acclimatized at four acclimation temperatures (22, 26, 30 and 34°C) for 30 days before starting the trial. After that, both CTMax and CTMin tests were conducted by gradual increase or decrease in water temperature at the rate of 0.3°C per minute of each test chamber set at acclimated temperature till fish start showing loss of equilibrium (LOE), i.e., unable to maintain dorsoventral orientation for ~ 1 min. The highest and lowest temperatures at which fingerlings of each strain showed LOE were observed and considered critical thermal maxima (CTmax) and critical thermal minima (CTmin) of a particular strain, respectively. A thermal tolerance polygon over the range of 22-34°C revealed comparatively the highest CTmax with the largest total and intrinsic polygon zones as well as the upper and lower acquired thermal tolerance zones of TMs followed by WRs, however IBDs showed significantly the highest CTmin value and the smallest intrinsic and acquired thermal tolerance zones. Furthermore, this study also investigated the expression of heat shock protein genes in response to thermal shock

by subjecting low (13°C) and high (37°C) thermal stress to 26±1^{o}C acclimated fingerlings of all strains. Results indicated the highest mRNA levels of HPS70 and HPS90 genes in the TMs, followed by WRs groups exposed to normal (26°C) and the highest temperature (37°C) stress. However, the IBDs followed by CMs showed the highest expression of both genes after exposure to a low temperature. The current study also evaluated the comparative disease resistance of all strains by injecting the pathogen *Aeromonas hydrophila (ATCC49140)* at a concentration of 1.7 × 104 CFU/g body weight and monitoring the fish for any disease symptoms and mortality. The post-challenge survival rate showed a similar trend, i.e., the highest survival % of the fingerlings of TMs followed by WRs, while fingerlings of CMs and MKs showed the highest mortality rate. The post-challenge haematological indices: RBC, HB, WBC, platelets, hematocrit, and innate immunity parameters: total protein, IgM, lysozyme, respiratory burst activity, phagocytic activity, and phagocytic index also showed the highest values in the fingerlings of TMs while CMs showed the lowest values. The spatial and temporal relative expression of immunity-related genes: Complement component (*C9),* Transferrin and Hepcidinafter challenge to *A. hydrophila* were in accord to the results of immunity indices and indicated a significantly higher mRNA level of these genes in the liver and muscle tissues of the fingerlings of TMs followed by WRs and MKs while CMs showed comparatively lower expression. The overall results of this study provide the baseline data for the initiation of the genetic improvement program and indicate the genetic diversity and variation in growth performance, thermal tolerance, innate immunity, and disease resistance among different wild and captive-bred strains of *L. rohita*. In conclusion, TMs exhibited more vigour in terms of growth rate, stress tolerance and disease resistance, and could be suggested for improving fish production.

INTRODUCTION

Genetic diversity, crafting the detailed elements that shape the biological world, is evident even within maternal twins, who share a womb and a remarkable bond, with distinctions emerging and emphasizing the prevalent nature of variability.It is represented by variability in traits among organisms and is fundamental for the stability and development of fish populations. This diversity plays a pivotal role in upholding the biological potential and overall health of fish stocks (Oster et al., 1988; Tave, 1999; Chauhan and Rajiv, 2010). The evaluation of genetic variation involves a detailed exploration of the gene count within a specified pool of genes, a process that unfolds dynamically and contributes to the continually evolving genetic diversity (DWR, 2014; CRRI, 2014; Ahmad et al., 2022).

Primarily originating from fundamental cellular processes and environmental interactions, genetic variation derives its initiation from the sexual recombination phenomenon occurring during meiosis. This dynamic process gives rise to the crossing over of homologous chromosomes, an event that can be influenced by an array of factors, including selection, mutation, migration, and genetic drift (Chauhan and Rajiv, 2010).While environmental variations and shifts in population density contribute to shaping variability, the continuous dynamics of genetic drift, migration, selection, and human intervention hold paramount significance in this complex process (Chauhan and Rajiv, 2010; Perez et al., 2012).

The dynamic interaction of various factors leads to continuous fluctuations in the allele frequency and genetic diversity of populations. Specific alleles emphasized through processes such as domestication or deliberate artificial selection, gain prominence, thereby increasing their prevalence within the genetic makeup (Yilmaz and Boydak, 2006). Fish faunal diversity encompasses the range of alleles or genotypes existing within a fish population and the diverse array of fish species in aquatic environments (Naish and Hard, 2008). Populations with increased genetic diversity, often associated with larger sizes, exhibit enhanced resilience against the unpredictable forces of genetic drift, which indiscriminately influences genetic variation (Lu et al., 2014).

Genetic improvement is crucial for individuals or species aiming to maintain a particular variation in their gene pool (ICAR, 2007). Genetic programs in aquaculture serve as systematic approaches,

aligning with the inherent diversity within fish genetic resources. These programs generate new strains with desirable traits, such as resistance against diseases, thermal tolerance, and improved survival and hatchability (Xia et al., 2014).

Aquaculture has utilized the adaptability of various fish species to meet the growing protein demand, especially in providing a nutritionally balanced diet for the expanding global population. With the challenges posed by climate change, there is an increasing emphasis on cultivating strains demonstrating resilience to rapidly changing environmental conditions. (Xia et al., 2015; Bhandari et al., 2017). The cumulative impact of a growing human population, rising seafood demands, limitations on freshwater resources, and the depletion of marine fish populations exert substantial pressure on aquaculture production (Lotze et al., 2018). This necessitates a paradigm shift, calling for increased seafood production with reduced water consumption and condensed timelines, prompting the adoption of efficient and predictable production processes. These processes, in their implementation, require the utilization of selectively bred improved lines possessing enhanced performance attributes. Despite the vast diversity of over 32,000 fish species, the majority remain untapped for mid to large-scale production, underscoring the urgent need for comprehensive genetic programs designed to meet the specific requirements of culturable species. Traditional methods may no longer be adequate to meet the evolving demands of a dynamically changing world.

Numerous successful genetic enhancement programs have been previously conducted. An important study by Norwegian breeding programs during the 1970s focused on salmonid fishes, revealing the potential for a 13-14% increase in growth per generation through selective breeding and improved feed conversion efficiency in comparison to wild stocks (Thodeson et al., 1999). ICLARM launched the "Genetic Improvement of Farmed Tilapia (GIFT)" project from 1988 to 1997, partnering with the Philippine and Norwegian Institute of Aquaculture Research. This project, focused on enhancing breeds for aquaculture, demonstrated remarkable results. Within just one generation of selection under different conditions, the GIFT project showcased notable advancements in tilapia breeds' growth and survival rates. The 7th generation of the enhanced GIFT line displayed an impressive 77% increase in growth compared to local tilapia strains in the Philippines, as documented by Eknath in 1992 and Eknath et al. in 1998. Acknowledging the prospective advantages inherent in selective breeding, Vietnam, along with other major carp-producing nations in Asia like Bangladesh, China, India, Indonesia, and Thailand, launched GIFT-type research programs. These initiatives aimed to

genetically enhance five carp species, driven by the pressing need for sustainable productivity in cultured carp stocks (Gupta and Acosta, 2001). Reports indicate that a selective breeding program can achieve a 7% growth increase per generation for rainbow trout (Kause et al., 2005), while Coho salmon *(Oncorhynchus kisutch)* demonstrated a weight increase of more than 60% (Hershberger et al., 1990) and advanced spawning by 13–15 days (Neira et al., 2006) after four generations of selective breeding. Similarly, channel catfish, in response to selection, displayed an approximately 80% improvement in growth, averaging a 13% increase per generation (Dunham et al, 2008). Important findings emerged from a thorough evaluation of the possible combined impact of improved fish breeds in five Asian countries: Bangladesh, China, the Philippines, Thailand, and Vietnam. Adoption of improved strains led to increased fish consumption among consumers who do not produce fish, decreased fish costs, increased profitability in fish farming, and significant effects on each nation's entire economy(Mohan et al., 2019; Kamran et al., 2023). These countries employed a combination of strain selection, selection, and/or crossbreeding in their respective initiatives.

Pakistan is endowed with an abundance of water bodies, including rivers, streams, canals, lakes, drains, dams, flood regions, and so on. However, despite this abundance of natural resources, the fishing industry only accounts for 1% of GDP and only 3.5% of agricultural production. Furthermore, among fishing nations, Pakistan ranks 28th position in terms of export earnings. Inland aquaculture in Pakistan primarily consists of Indian major carp which are widely cultured in Asian countries and have great economic importance (Ahmed et al., 2008; Shah et al., 2011; Kamran et al., 2023). However, in countries like Pakistan, in a competition to produce more and more seeds to bridge the gap between demand and supply, and due to inadequate knowledge, most fish farmers use subpar strains and show little interest in genetic quality. (Hussain and Mazid, 2005). Consequently, the carp industry faces several problems like high mortality, poor growth, deformity, etc., these may be due to inbreeding depression, poor strain selection, or due to deterioration in genetic quality (Ponzoni et al., 2007).

In the last decade, Pakistan has made significant efforts to increase the production of crops, livestock, and poultry through genetic tools like strain evaluation, selective breeding, crossbreeding, hybridization, etc., and breeding techniques. However, fish, despite their excellent nutritional value and substantial contribution to the diet, have not reaped the benefits.Therefore, most of the currently farmed species in Pakistan are based on poor strains. Although there is a great gap between the global

supply and demand of fish in Pakistan, this difference is much more pronounced and only 2.0 kg/capita/annum is available as compared to America, China, and other countries where it is consumed at the rate of 11-18.4 kg/capita/annum (FAO, 2011). The anticipated supply and demand of fish can be balanced by aquaculture, but only if improved strains and breeds are created by the application of a genetic improvement program as the lack of genetic improvement programs results in seeds that are associated with low survival, slow growth rate, deformity, sensitive to disease, etc.(Shah et al., 2011). In general, high-quality seed is essential for successful fish cultivation and the long-term development of the aquaculture system.

Strain selection in fish breeding is favored over hybridization due to its simplicity, cost-effectiveness, and ease of implementation, particularly for individual farmers or small-scale operations. Hybridization, involving the crossing of individuals from different strains, can be complex and resource-intensive, making it less practical for those with limited resources or technical expertise. Moreover, hybridization may not guarantee stable expression of desirable traits across generations, posing challenges for consistent trait inheritance. In contrast, strain selection allows for a streamlined breeding process within a specific genetic strain, ensuring the preservation and reliability of desired traits over time. This method is farmer-friendly, enabling a more accessible and manageable approach to selective breeding, while also facilitating adaptability to local environmental conditions.

The selection of strains for genetic improvement programs is a crucial step, influenced by factors such as geographical distance and the resulting genetic diversity. According to studies (Gould et al., 2023; Rahman et al., 2009), strains, defined as genetic variants within a species, are often shaped by the intent for genetic isolation. This concept of strains, as articulated by Ten over et al. (1995),involves isolates distinguished by phenotypic or genotypic characteristics. In practical terms, utilizing established and high-performing domestic strains is the initial and expedient strategy for initiating genetic improvement (Dunham, 2023; Kool boon et al., 2014). Fish strains, such as the channel catfish (*Ictalurus punctatus*) and rainbow trout (*Oncorhynchus mykiss),* display notable diversity in characteristics including growth rate, susceptibility to diseases, and body morphology and reproductive characteristics (Dunham and Smitherman, 1984; Kincaid, 1981). This emphasis on strain selection underscores the significance of leveraging inherent genetic diversity to achieve targeted improvements in fish populations.

The rohu (*L. rohita*) is a significant freshwater fish species in South Asian rivers, recognized for its taste and high market demand. It is an omnivorous fish widely used in aquaculture and prevalent in the rivers of northern central and eastern India, Pakistan, Vietnam, Bangladesh, Nepal, Myanmar, Peninsular India, and Sri Lanka (Dahanukar, 2010; Froese et al., 2017). The species exhibits specific food preferences throughout its life stages, starting with zooplankton during early development and shifting to an herbivorous diet as a juvenile or adult. Rohu, reaching sexual maturity between two and five years, spawns during the monsoon season in flooded rivers. The fish's spawning season aligns with the southwest monsoon, and spawn collection is common for tank and lake rearing (Froese et al., 2017). A crucial aquaculture species in South Asia, particularly in Pakistan, *L. rohita* is renowned for its taste and high market demand (FAO, 2018). Profitable farming, with a production period of at least 8 months, caters to the substantial market demand, making it a lucrative venture. Cultivated for 10-12 months, *L.rohita* reaches a size of 700-800 g, with well-prepared ponds facilitating even greater growth. Harvesting aligns with market demand, with the fish being particularly sought after when weighing 1 to 2 kg. In Pakistan*L. rohita* is integral to local dishes like fish fry and fish curry, contributing to its substantial demand. With a market price ranging from Rs. 300 to 450 per kg, rohu farming, especially when combined with Catla and Mori fish, can yield significant revenues for farmers—up to Rs. 1.5 lakhs per acre annually. This traditional farming practice dominates approximately 80% of the total farming area in Pakistan, showing*L. rohita* popularity among consumers (Rahman, 2005).

However, over the past two decades, the density of the wild species has declined considerably because of multiple factors like lower recruitment, indiscriminate exploitation, overfishing, destruction of habitats, blockage of migratory routes, and interruptions by human (Okumusand Ciftci, 2003; Islam and Alam, 2004; Lu et al., 2014) and its contribution in seed production became less than one percent in recent times (Simonsen et al., 2005). The wild population of *L. rohita* has experienced a decline in natural seed production, stemming from historical collection practices where seeds were primarily sourced from rivers. Consequently, this decline has led to a reduction in the wild rohu population's contribution to less than one percent in contemporary times.In such a scenario due to continuous utilization of the same broodstock, inbreeding is a common phenomenon in private hatcheries (Simonsen et al., 2005). Poor performance of the stock developed through using such seeds has increased the incidence of diseases and mass mortalities have been reported in recent years.

The destruction of wild stocks that are genetically distant puts the wild population of *L. rohita* at a high risk of losing genetic variability (Luhariya*et al.*, 2012). Moreover, the poor performance of the hatchery stock may also indicate the loss of genetic variability among hatchery-reared fish compared to wild counterparts (Arakiand Schmid, 2010) because of limited brood stock andcontinuous utilization of the same broodstock generation after generation (Araki and Schmid, 2010) as noted in some studies that over the past two decades, The decline in genetic diversity among wild fish populations is attributed to numerous factors, including diminished recruitment rates, unselective exploitation, overfishing, habitat degradation, impediments to migratory pathways, and human intervention (Okumus and Ciftci, 2003; Islam and Alam, 2004; Lu *et al.*, 2014). *L. rohita* is a valued fish that needs to be cultured keeping the ever-growing demand in mind as traditional methods of culturing result in lower production and the restocking programs for the replenishment of *L.rohita* stock in various rivers of the country are causing genetic pollution (Qadeer and Abbas, 2017) which demands immediate measures for the genetic improvement of this species while maintaining its genetic integrity.

In Pakistan, the artificially induced *L.rohita* has been cultured in different geographical regions such as Punjab, Khyber Pakhtunkhwa, Sindh, and Baluchistan etc., in different closed ponds. Sometimes they are reared and used as brood fish for breeding programs, but these fish may come from the same brood population or same tributary. It is well documented that for sustainable development of fisheries and aquaculture, there is a great need to maintain genetic quality but unfortunately in the past little attention has been given to it (Hasanat *et al.*, 2015). In Pakistan, scanty information/literature is available on genetic variability in indigenous major carp including *L. rohita*, Moreover, due to conventional breeding techniques, there is a chance/risk of inbreeding depression.

To mitigate the inbreeding depression, some hatchery owners catch wild carp from the river and breed them with their brood stock to compensate for this negative effect. This reduces inbreeding in the population while also negating any beneficial effects of natural selection in the ponds and selection for growth and other desirable traits (Gjerde et al, 2005). Maintaining genetic quality is critical for the sustainable development of fisheries and aquaculture.

It is a well-established fact that for any genetic study, it is important to evaluate the existing strains from wild and hatchery-reared populations for genetic variability which will help in Enhancing

comprehension regarding the historical and contemporary mechanisms influencing the genetic composition of *L. rohita*. For this purpose, strain evaluation for the measurement of genetic diversity is of utmost importance. For this reason, the main goals of any fish genetic modification programme are to find different strains (if available), create diversity (if not available or limited), and then take advantage of it. Comprehensive knowledge of genetic variety in all its dimensions is crucial within this framework *viz*., Factors influencing genetic diversity, various methods of diversity analysis, their measurement, and statistical analysis software become necessary to build a baseline population for the commencement of a genetic program (Nousias et al., 2021). In a general sense, genetic diversity serves as the foundational reservoir for natural selection, enabling organisms to undergo adaptations suited to novel and demanding environmental conditions. (Bernatchez et al., 2017; Gandra et al., 2021).

Initially, analysis of genetic diversity was carried out through morphological, cytological, and biochemical characterization but due to controversial results, more advanced techniques utilizing genomic tools such as microsatellite and mitochondrial DNA (mtDNA) markers became the preferred method. These markers have several properties like abundance, better genomic coverage, high reproducibility, phenotypic neutrality, co-dominance and lack of environmental fluctuations (Cholastova and Knotova, 2012; Mondini et al., 2012; Basavaraju et al., 2014; Figueras et al., 2016), therefore, widely used in aquaculture for genetic improvement programs and, conservation and management of natural resources (Liu and Cordes, 2004).

Molecular markers, associated with specific loci, are vital for identifying sequences within an unknown genome. Polymorphism, arising from mutations like insertion, deletion, duplication, inversion, or point mutation, results in differing base compositions at individual loci. Various techniques, including PCR-based markers such as AFLP, RAPD, ISSR, and SSR, are employed in biology and biotechnology to assess polymorphism (Schork et al., 2000).

In *L. rohita* studies, both RAPD and microsatellite markers have indicated molecular marker differentiation (Islam and Alam, 2004; Alam et al., 2009; Sahoo et al., 2014; Ullah et al., 2015; Qadeer and Abbas, 2017). *L. rohita* has 50 chromosomes and approximately 1950 Mb nucleotide base pair number in its genome (Patel et al., 2009).

Microsatellite markers are pivotal along a species' distribution range, revealing population structures influenced by environmental and behavioral factors (Adams and Rosel, 2006; Hoelzel et al., 1998; Lázaro et al., 2004; Lessios, 2008; Sellas et al., 2005). Population dynamics are explored through mtDNA and microsatellite markers (Baker et al., 1998; Costa-Urrutia et al., 2012; Escorza et al., 2000; Lyrholm et al., 1999; Méndez et al., 2010; Natoli et al., 2005; Natoli et al., 2008; Pope et al., 1996; Tonione et al., 2011).

Genetic marker development significantly impacts various biological areas, including aquaculture, offering insights into genetic variability, parentage relationships, and breeding program performance (de León et al., 1998; Spidle et al., 2004; Vandeputte et al., 2004; Romana-Eguia et al., 2004; Liu and Cordes, 2004). Markers like allozymes, AFLPs, ESTs, microsatellites, mtDNA, RAPDs, RFLPs, and SNPs are now available for diverse aquaculture species (Liu and Cordes, 2004).

Microsatellites are an essential tool in genetic study because of their strong polymorphism, cross-species conservation, and wide genomic coverage. They have repeated arrays of mono-, di-, tri-, and tetranucleotide sequences. (FitzSimmons et al., 1995; Pépin et al., 1995; Goldstein, 1999; Waldbieser et al., 2001; Cunningham and Meghen, 2001). Widely used in aquaculture genetics, microsatellites enable intrapopulation and interpopulation comparisons, strain identification, and assessment of genetic variability (Abdul-Muneer, 2014; Wattanadilokchatkun et al., 2022). However, due to the ambiguity of ancestral information, it cannot be utilised to infer genealogical patterns of relationships (Zhang andHweitt, 2003). Using both sorts of markers, we can better understand genetic diversity and relatedness among species or populations (Gariboldi et al., 2016). A microsatellite, also known as a simple sequence repeat (SSR), is made up of numerous rounds of tandemly organised repeats of one to six nucleotides in length. Microsatellites, being co-dominant, represent the most versatile molecular markers (Tautz, 1989). Despite the time-consuming nature of identifying microsatellites in a species of interest, the employed techniques are well-established and reliable. Microsatellite markers have proven to be highly effective in identifying genetic variation both within and between populations of various aquatic species, including fisheries-related vertebrates and invertebrates (Aranishi and Okimoto, 2005; Kim et al., 2013). However, the most common issues of microsatellite markers include the necessity for species-specific primers and the potential requirement for a substantial number of microsatellites to achieve adequate statistical power, the labor-intensive and costly nature of experiments has been noted (Olafsson et al., 2010). To mitigate this issue, the cross-

species amplification technique is employed to improve cost-effectiveness. The success rate of cross-species microsatellite amplification depends on the evolutionary relatedness of isolated loci and the species to which heterologous loci are applied (Primmer et al., 1996). A small number of highly polymorphic microsatellites may suffice for addressing inquiries related to genetic diversity. Although the population genetic structure of mrigal fishery has been characterized in only a few studies, one study in the Himalayan region utilized both microsatellites and allozymes (Chauhan et al., 2007), while others utilized mitochondrial Cyt b gene, ATPase-6 gene, and truss morphometric (Das et al., 2014; Behera et al., 2015). Microsatellites are ubiquitously present in the euchromatic regions of genomes, exhibiting high levels of polymorphism and apparent neutrality, and are inherited biparentally. Their integration into population genetic studies has enhanced our ability to assess genetic diversity, parentage, relatedness, fine-scale population structure, and recent population history. However, the inference of genealogical relationships is hindered by the inherent ambiguity in the ancestral information contained within microsatellites (Pope et al., 1996; Zhang and Hewitt, 2003). Microsatellites, due to their high polymorphism and biparental inheritance, are widely employed as markers for investigating geographical structure and gene flow (Jarne and Lagoda 1996). These markers, inherited according to Mendelian principles, provide an efficient method for examining multiple nuclear loci to assess geographical variation in natural populations, akin to allozymes. However, the analysis of microsatellite data encounters challenges stemming from their intricate mutation processes (Palsbøll, 1999; Rubinsztein et al., 1999), the difficulty in distinguishing alleles that are identical-by-descent from those that are identical-by-state (Estoup et al., 2002), and their typically high mutation rates, which may result in the underestimation of differentiation among populations (Hedrick, 1999). Comparative investigations in avian populations, contrasting results from microsatellites and mtDNA, frequently indicate reduced nuclear genetic structure (Helbig et al., 2001; Crochet et al., 2004; Eggert et al., 2004). Nevertheless, exceptions have been noted, demonstrating either similar levels of genetic structure (Burg and Croxall, 2001) or higher levels of nuclear structure (Johnson et al., 2004).

Molecular markers, extensively utilized in fisheries studies (Akter et al., 2010), play a crucial role in identifying genetic diversity and conserving fish populations (Almeida et al., 2001). Various DNA markers, A range of molecular methods, including RFLP, AFLP, RAPD, and microsatellites, can be

applied to maintain piscine genetic diversity, identify pure lineages, and identify genetic changes in both wild and cultured strains of a particular fish species (Liu and Cordes, 2004).

According to Weersing and Toonen (2009), these DNA markers are widely used in freshwater and marine fish species to record genetic diversity and demographic histories. Microsatellite markers are widely used and a useful instrument for characterising and assessing genetic diversity. They are made up of brief, tandemly repeated sequence motifs, the length of the repeat units varying from one to six base pairs. Because detecting variations in repeat numbers by PCR is so easy, simple sequence repeats (SSRs), sometimes referred to as microsatellites, have been used as genetic markers in many different species (Tautz and Schlotterer, 1994; Moore et al., 1991).

Microsatellite markers play a pivotal role in elucidating genetic diversity among different strains of the same species and in detecting variations within both wild and cultured populations (Was and Wenne, 2002). For instance, Das et al. (2005) conducted a study that developed twelve microsatellite markers specifically tailored for *L.rohita*. The investigation aimed to evaluate the polymorphism of these markers within farm-raised *L. rohita* populations in India. Similarly, Alam et al. (2009) examined the population genetic structure of *L.rohita* in Bangladesh, utilizing four di-nucleotide microsatellite loci (Lr3, Lr12, Lr14b, and Lr21). Additionally, Saha et al. (2010) scrutinized the population genetic structure of an endangered carp, *Labeocalbasu*, sourced from three distinct stocks (the Jamuna River, the Halda River, and a hatchery in Bangladesh). This analysis involved the utilization of four heterologous microsatellite loci (Lr12, Lr14b, Lr21, Lr24), originally identified from *L.rohita*.

In contrast, despite the reliability of microsatellite markers in assessing genetic diversity, the aquaculture sector has recently shifted its focus to mtDNA analysis for evaluating deep divergence among populations or species. This shift is understandable due to the numerous benefits of mtDNA, including easy collection, inheritance to the progeny, lack of recombination, and a high rate of base substitution resulting from the accumulation of divergence sequences (Brown, 1985). MtDNA is assuming significance in aquaculture for identifying significant differences among populations or species (Suneetha et al., 2000; Apostolidis et al., 2008; Wu et al., 2010; He et al., 2011; Mandal et al., 2012). Due to its simplistic collection, uniparental inheritance, mtDNA has been widely used as a marker in phylogeographic investigations due to its high rates of base substitution and lack of recombination (Avise, 2012; Dowling et al., 1996; Pope et al., 1996; Tonione et al., 2011).

Numerous researchers have advocated DNA barcodes, based on a specific mitochondrial segment containing 600 base pairs recognized as cytochrome oxidase I (COI), as a swift, efficient, and cost-effective technique for evaluating genetic diversity and relatedness among species or populations (Hebert et al., 2004). However, nonetheless the various advantages, it is important to acknowledge that population studies exclusively reliant on this locus might exhibit bias towards processes mediated by females due to its maternal inheritance (Avise, 2012; Pope et al., 1996; Zhang and Hewitt, 2003). Despite reservations raised by several researchers concerning population studies solely centered on mtDNA owing to its maternal inheritance (Avise, 2012; Pope et al., 1996; Zhang and Hewitt, 2003), Genetic differentiation has been found to be much higher when quickly changing markers, like the mitochondrial regulatory region, are applied (Zink et al., 1997). The difference between allozymes and mitochondrial results, according to Crochet (2004), could result from differences in the effective population size of these markers. This means that similar degrees of gene flow could produce the F-statistic distributions that are frequently seen for these markers. Significant geographic structure predicted from mtDNA is found in many cases, however these are typically explained as the result of recent range expansions and non-equilibrium populations (Zink et al., 2003).

Given the multiple benefits provided by both approaches, it is critical to apply them in conjunction. The importance of assessing concordance among distinct genetic markers has been widely debated in scientific literature (Avise, 2000). Fresh datasets can independently support or refute previously established hypotheses. Simultaneous study of many loci can reduce variability in estimated parameters due to random sampling and lineage sorting effects (Takahata, 1989; Hudson, 1990). Divergent outcomes across markers, especially those with diverse modes of inheritance, such as microsatellites and mtDNA, can reveal novel insights that are not possible with single data types (Prugnolle and Meeus, 2002). Inconsistent patterns across loci might result from a variety of factors. Molecular evolutionary parameters linked with the chosen markers, such as effective population size and evolutionary rates, can have a major impact on the capacity to identify genetic diversity and geographical organisation. For example, the identification of significant genetic structure in mtDNA is frequently due to its small effective population size, which makes it more sensitive to random drift effects (Haavie et al., 2000). Evolutionary rates play an important effect in detecting ability. Slow evolutionary rates may result in insufficient polymorphism to accurately characterise existing genetic structure, whereas high rates may mask indications of significant differentiation, which is frequent with microsatellites (Hedrick, 1999). Molecular incompatibilities between genome groups can

provide diverse results, especially if they cause unequal introgression of markers across hybrid zones (Fel-Clair et al., 1998; Payseur et al., 2004). Finally, ecological factors influencing organism behaviour, such as sex-biased dispersal and assortative mating, can have a significant impact, especially when markers are gender-specific (Prugnolle and Meeus, 2002). In avian studies, incongruous patterns are frequently attributed to sex-biased dispersal, which aligns with behavioural observations (e.g., male-philopatry and female-biased dispersal in red grouse) (Piertney et al., 2000), but they can also reveal unexpected results that challenge conventional wisdom (Gibbs et al., 2000). Furthermore, disparities between markers are frequently associated with the presence of hybrid zones, resulting in differential introgression of mtDNA and nuclear markers (Hansson et al., 2006) or reduced fitness of hybrid females (in avian heterogametic sex), as predicted by Haldane's rule (Helbig et al., 2001).

After examining both microsatellite and mitochondrial markers for the genetic diversity study in *L. rohita*, it is crucial to shift the focus toward the consideration of pertinent breeding parameters. Exploring these factors will not only complement the diversity findings but also provide confirmation and deeper insights into the genetic dynamics of the studied population. The sustainability and profitability of fish farming, along with the expansion of aquaculture in any country, are well-established to be entirely dependent on the sufficient supply of quality fish seed for culturable species (Islam et al., 2007). However, fish seed production depends on various factors, including management practices and the genetic makeup of broodfish. The ability of a fish species to produce offspring to ensure the continuation of its generation is referred to as breeding performance. When choosing a strain, one crucial step in ensuring that only the best strain in the program is propagated is to take breeding performance into account (Ohta et al.,1997). The quality and quantity of the next generation is highly dependent upon the quality of the brooder. However, the reproductive performance of the brooder is influenced by genetic and environmental factors (Dupont-Nivet et al., 2008). Furthermore, genetic vigour is a life history attribute that is highly relevant to fisheries management since it is thought to be a crucial component in explaining differences in population size, recruitment, and population growth rate (Kader, 1984). It varies between species and individuals and has been linked to environmental factors, genetics, brood size (length, weight, and age), and availability of food. The enhancement of fish production depends very much on the health of fry and fingerlings used for rearing and careful and scientific maintenance is thus required.

The paramount factor influencing farmed fish production is growth performance, a critical aspect of genetically enhancing various fish species to increase production sustainably in ponds and other culture systems. Fish growth is a multifaceted phenomenon influenced by both genetic factors and environmental conditions (Pepin, 1991; Handeland et al., 2008; Kamran et al., 2023). Among these factors, temperature plays a crucial role in regulating fundamental biological processes. It affects fish growth and survival rates (Pepin, 1991; Kupren et al., 2011), metabolic activities (Sandersfeld et al., 2017), and embryonic development (Thepot and Jerry, 2015).

Despite the recognized contribution of environmental factors, several investigations suggest their influence to be 40% or less in fish development under natural circumstances (Wilson and Meekan, 2002; Caldarone et al., 2003; Green and McCormick, 2005). This underscores the predominant effect of genetic factors on the phenotypic traits of fish. Sexual reproduction contributes to the uniqueness of each fish genetically, fostering essential genetic variation crucial for successful adaptation to environmental changes and ensuring the species' survival (Ryman et al., 1995). This inherent diversity forms the foundation for selecting optimal strains or breeds for subsequent generations. Concurrently, certain strains have undergone hormonal sex reversal, interspecific hybridization, and genetically male population technologies to address challenges such as early sexual maturation and overpopulation in fishponds. These technologies, as mentioned earlier, may impact phenotypic traits and environmental fitness.

Many fish strains exhibit a great deal of variation in traits such as growth rate, coloration, resistance to disease, body form, proportion of dressing, maturation age, timing of spawning, and fecundity (Newton et al., 2010; Mohsin et al., 2015). For example, different strains of *O. mykiss* rainbow trout display differences in vigilance against infectious pancreatic necrosis (IPN), while different strains of Barramundi (Lates calcarifer) display differences in tolerance to upper temperature limits, swimming balance, and caudal fin characteristics (Newton et al., 2010).

Consideration of the growth performance is among the core goals of any genetic improvement program which is reflected in the highly successful "Genetic Improvement of Farmed Tilapias" (GIFT) project, led by ICLARM (currently WorldFish), that significantly contributed to elevating tilapia production in Asia and the Pacific region. Initiated nearly 30 years ago, this collaborative research initiative aimed to develop a high-performing farmed tilapia strain adaptable to a broader range of environments through selective breeding practices. Over the course of six generations, the

project showed consistent improvements of 10-15% every generation. with the current GIFT strain exhibiting a remarkable 100% faster growth than the founder stock, gaining widespread global popularity. Similarly, selective breeding approaches have successfully enhanced various fish species, influencing cultivation environments globally.

Despite the genetic makeup of various strains, the presence of genotype by environment interaction (G × E) necessitates careful consideration. A strain optimized in one environment may not perform equally well in less favorable conditions, underscoring the importance of assessing species' performance under variable conditions with varying human interventions to obtain an accurate representation.

Assessing the capabilities of different strains across diverse conditions is crucial. Equally important is confirming growth performance through molecular tools and examining the expression of genes regulating hunger and growth, with variable expression across strains being a key observation. Feed intake regulation primarily involves hormones linked to hunger and growth. The peptide hormone ghrelin plays a pivotal role in regulating food intake and metabolism. It is a strong inducer of food consumption, leading to elevated body weight and obesity in mammals. Ghrelin stimulates the release of growth hormone in a variety of species, including teleost fish such as sea bream, rainbow trout, tilapia, and channel catfish, as well as mammals, birds, and amphibians (Rønnestad et al., 2017). In fish, ghrelin exhibits species-specific effects on food intake (Kang et al., 2011), and evidence shows ghrelin expression induced in *L. rohita* fingerlings during starvation (Dar et al., 2018).

Abiotic and biotic factors, along with growth rate, represent significant factors influencing the economic viability of commercial fish farming (Dar et al., 2018). Insulin-like growth factor-I (IGF-I) modulates the secretion and functionality of growth hormone. While IGF-1 primarily regulates growth hormone secretion and influences overall growth, IGF-2 plays a significant role in fetal development and has been associated with cell proliferation and differentiation. (Pandey et al., 2009). In numerous fish species, A positive association has been observed between dietary intake level, dietary protein concentration, and the growth rate of the body, and the expression of mRNA encoding insulin-like growth factor-I (IGF-I) and insulin-like growth factor-II (IGF-II) (Huang et al., 2016).

Similarly, it is crucial to assess the expression of myogenin and MyoD in various strains. Fish skeletal muscle growth is significantly regulated by myogenic regulatory factors (MRFs) and

myostatin (Johnston et al., 2009). The expression of MyoD, myogenin, and myostatin is necessary for fish muscle development to proceed. Moreover, a variety of nutritional variables, specifically amino acids, which are the building blocks of proteins, may influence these growth-related activities (Machado et al., 2019).

Myogenic regulatory proteins, including MyoD and Myf5, govern the initiation and proliferation of satellite cells, while Mrf4 and myogenin contribute to cell differentiation (Watabe, 2000). Additionally, myostatin acts as a suppressor, impeding the growth and differentiation of muscle cells, thus impeding myogenesis. (Lee et al., 2009).Understanding these interconnected processes is essential for comprehending the dynamics of fish muscle growth and development.

With the consideration of growth performance in various setups, it is also very important to consider other aspects of the growth performance while selecting strains for genetic improvement because in response to global climate change, which poses a significant threat to biodiversity and the aquaculture sector, impacting fish productivity worldwide (Ficke et al., 2007), fish exhibit phenotypic plasticity and adaptability through acclimation and physiological modifications (Schulte et al., 2011; Fox et al., 2019). However, each species has a different level of physiological flexibility in terms of heat tolerance and strains, influencing their adaptation (Stitt et al., 2014; Fu et al., 2018). Understanding the thermal tolerance of aquatic ectotherms is crucial, as excessive, and suboptimal temperature levels can induce metabolic stress, impact immunity, and increase susceptibility to pathogens (Claësson et al., 2016; Dominguez et al., 2004; Abram et al., 2017; Liang et al., 2014). Apart from knowing the phenotypic traits, it is also important to understand that a comprehensive understanding of the physiology of a fish necessitates the acknowledgment that its observable traits may not always provide an accurate representation. Consequently, a molecular-level investigation becomes crucial to determine the authenticity of its underlying physiology, which can be validated through a meticulous analysis of gene expression patterns associated with stress (Boamah et al., 2022). In this context, heat shock protein (HSP) genes, known for their role in protein homeostasis and stress response, become important molecular biomarkers for studying stress responses in fish (Lindquist, 1986; Chaudhury et al., 2021; Sen and Giri, 2017). These genes are classified into various families, including HSP100, HSP90, HSP70, HSP60, HSP40, and smaller HSP groups. Among these families, HSP70 and HSP90 are of key importance regarding thermal stress (Rehman et al., 2021) playing a crucial role in

maintaining cellular functioning and protecting against temperature-induced cellular damage (Archana et al., 2017; Das et al., 2015; Somero, 2020; Shan et al., 2020).

The importance of these heat shock proteins in the case of *L. rohita* is increased by the fish's high vulnerability to hemorrhagicsepticemia, a common and extremely harmful bacterial infection brought on by the Gram-negative bacterium *Aeromonas hydrophila*, which becomes contagious during stressful events(Nayak et al., 2022).

With the assessment of heat shock proteins, it is also very important to consider the disease resistance of the strains. It is a handy approach for the selection of fish strains, involving the assessment of their disease resistance, a crucial factor in genetic improvement programs. Evaluating resistance, often measured as survival in challenge tests, provides a valuable means to determine the suitability of brood fish for such programs. Challenging various strains with specific pathogens, such as *A. hydrophila*, emerges as an effective method to gauge their resistance levels. This approach allows for informed decisions regarding the inclusion of strains in genetic improvement initiatives. Numerous studies have explored the potential of selective breeding to enhance disease resistance, presenting an opportunity to proactively address long-term disease challenges in aquaculture (Embody and Hayford, 1925; Ehlinger, 1964, 1977; Gjedrem and Aulstad, 1974; Refstie, 1982; Gjedrem, 1983; Bailey, 1986; Cipriano and Heartwell, 1986; Standal and Gjerde, 1987). Similarly, Gjedrem et al. (1991) illustrated considerable genetic diversity in susceptibility to furunculosis (*Aeromonas salmonicida*) within Atlantic salmon populations, thereby establishing a basis for the adoption of this approach. Recent investigations reaffirm the possibility of notable enhancements in disease resistance within diverse fish species, encompassing Atlantic salmon, rainbow trout, Atlantic cod, and Pacific white shrimp. This accumulating evidence underscores the genetic components influencing resistance to infectious diseases in aquaculture. While efforts have been made to identify intrinsic resistance factors and candidate marker traits, the complexity of these mechanisms suggests a multifactorial nature (Secombes andOliver, 1997). Despite challenges in identifying a single marker trait for efficient indirect selection, the exploration of genetic variations in disease response remains a promising avenue for advancing aquaculture practices.

Understanding the variable survival probabilities in the onset of the disease among different fish strains is of key importance for elucidating the adaptive capacity (Cooke et al 2016). In a variety of situations, the Kaplan-Meier approach has shown to be an effective tool for evaluating survival data and determining survival probabilities (Kristiansen et al., 2011). For calculating survival over time,

the Kaplan-Meier estimate is the most straightforward method. Every time, the survival probability is calculated by dividing the total number of fish at risk by the number of fish that survive.

By integrating survival probability analysis with the assessment of thermal tolerance and stress gene expression, a comprehensive understanding of the adaptive capacity and stress responses of different *L. rohita* strains can be achieved. This knowledge will provide valuable insights into the interplay between genetic factors, physiological responses, and environmental stressors.

A comprehensive evaluation of both immunological and hematological parameters is crucial for gaining a comprehensive understanding of fish strains' immune competence, disease susceptibility, physiological diversity, and overall health (Callaway et al., 2012; Magnadottir, 2010). By assessing immunological parameters such as AST levels, lysozyme levels, total protein levels, IgM levels, phagocytic activity, phagocytic index, and respiratory burst, researchers can understand the immune capabilities and vulnerabilities of fish strains (Saravanan et al., 2021). Concurrently, the examination of hematological parameters, including Hb, RBC count, platelet count, Hct, red cell distribution width (RDW), WBC count, and lymphocyte percentages, provides insights into physiological diversity, genetic variation, and health status (Neta et al., 2015).

Assessing the expression of key immunity genes, including Natural Killer Enhancing Factor (*NKEF*), transferrin, hepcidin, and complement component (*C9*), across diverse strains of *L. rohita* is crucial for the selection of the optimal candidates to kickstart a genetic enhancement program. These genes play pivotal roles in shaping the fish's immune responses and overall well-being. For instance, NKEF's involvement in enhancing natural killer cell activity is vital for bolstering innate immune defenses, a critical factor in ensuring robust disease resistance and overall fish health. Similarly, the evaluation of *C9* expression sheds light on the efficiency of the complement system, aiding in the identification of strains with heightened capabilities to combat microbial threats.

Moreover, the consideration of the hepcidin expression provides valuable insights into the regulation of iron homeostasis and the antimicrobial properties associated with this gene. Strains exhibiting well-balanced iron metabolism and effective defense mechanisms against pathogens, as indicated by hepcidin expression, are prime candidates for the genetic enhancement initiative. Additionally, the assessment of transferrin expression offers crucial information about iron availability for immune functions, enabling the selection of strains with a finely tuned iron transport system and a robust

immune response. Collectively, this comprehensive evaluation ensures the identification of strains with superior immune functionality, disease resistance, and overall fitness, aligning with the objectives of a genetic enhancement program in *L. rohita.*

It becomes evident that aquaculture production in Pakistan holds significant potential for enhancement through the implementation of a genetic improvement program. Unfortunately, to date, no such initiatives have been undertaken in the country. The application of straightforward genetic techniques, including strain selection, selective breeding, inbreeding, and crossbreeding, could elevate carp production by a substantial 7 to 13% in each generation. Among these methods, strain selection stands out as the most practical and accessible approach, especially in a country like Pakistan, where ample room for improvement exists. The imperative for a genetic improvement program is underscored by the need for advancements in the sector. Various scientists have actively engaged in genetic improvement programs for diverse fish species, employing a range of selection methods. Abundant evidence supports the notion that genetic tools can indeed be leveraged to augment fish production (Shah et al., 2012; Nielsen et al., 2010; Gjedrem and Baranski, 2009; Dunham, 2023; Neira et al., 2006; Storset et al., 2007). Consequently, Pakistan should start a thorough genetic enhancement programme with a primary focus on strain selection, is not only a viable strategy but also a crucial step toward optimizing aquaculture production and addressing the existing potential for growth in the sector.

Aim of the study

Establishment of a baseline population by the selection of superior strains from diverse geographical locations for incorporation into the genetic improvement program.

Objectives of the study

To accomplish the goal, the current study used several biochemical and molecular tools and conducted with the following objectives.

- To determine the genetic diversity within fish populations sourced from diverse geographical regions, by utilizing microsatellite and mitochondrial markers.

- To compare the growth performance of fry and fingerlings of different strains under controlled and semi-intensive culture system respectively.
- To compare thermal tolerance and thermal stress response of different strains of *L. rohita*
- To evaluate the health status, immunity, and disease resistance of different strains before and after challenge to pathogens.
- To compare the proximate composition of different strains after culturing in communal ponds.

MATERIALS AND METHODS

Experiment 1. Assessment of genetic diversity among wild and captive bred *Labeorohita* through microsatellite markers and mitochondrial DNA

For the evaluation of genetic diversity between various strains of *L. rohita*, fish were procured from six different Fish Hatcheries and two Rivers across the country. Rivers and Fish Hatcheries were selected based on their geographical location and diverse fish culture practices. The collection of strains was performed with the active collaboration of various collaborators at eight locations while local fishermen assisted in the collection of wild stocks from the River Ravi and Kabul Rivers. The strains along with the study area given in Fig 1 and for ease, the locations are abbreviated as follows:

- USs (Upper Sindh Fish Hatchery, Province, Sindh; 25° 23‘00” N 68°20‘05” E)
- SPs (Carp Hatchery and Training Center, Peshawar, Province, Khyber Pakhtunkhwa 34.0933° N, 71.5105° E)
- WKs (Wild Strain from River Kabul; 33° 55′0″N 72°13′56″E)
- WRs (Wild Strain from River Ravi; 31° 29' 2.2272"N 74° 9' 46.4292"E)
- TMs (Tawakkal Fish Hatchery Muzaffargarh, Province Punjab; 30° 11' 57"N 71°17' 07" E)
- MKs (Govt. Fish Hatchery, Mianchannu, Province Punjab; 30° 26' 20.1732"N 72° 21' 18.198"E)
- CMs (Govt. Fish Hatchery, Charbanda, Mardan, Province Khyber Pakhtunkhwa; 34° 21' 06" N 71° 58' 22"E)
- TKs (Tanda Govt. Fish Hatchery Kohat, Province Khyber Pakhtunkhwa33° 34' 07"N 71° 24' 01"E)

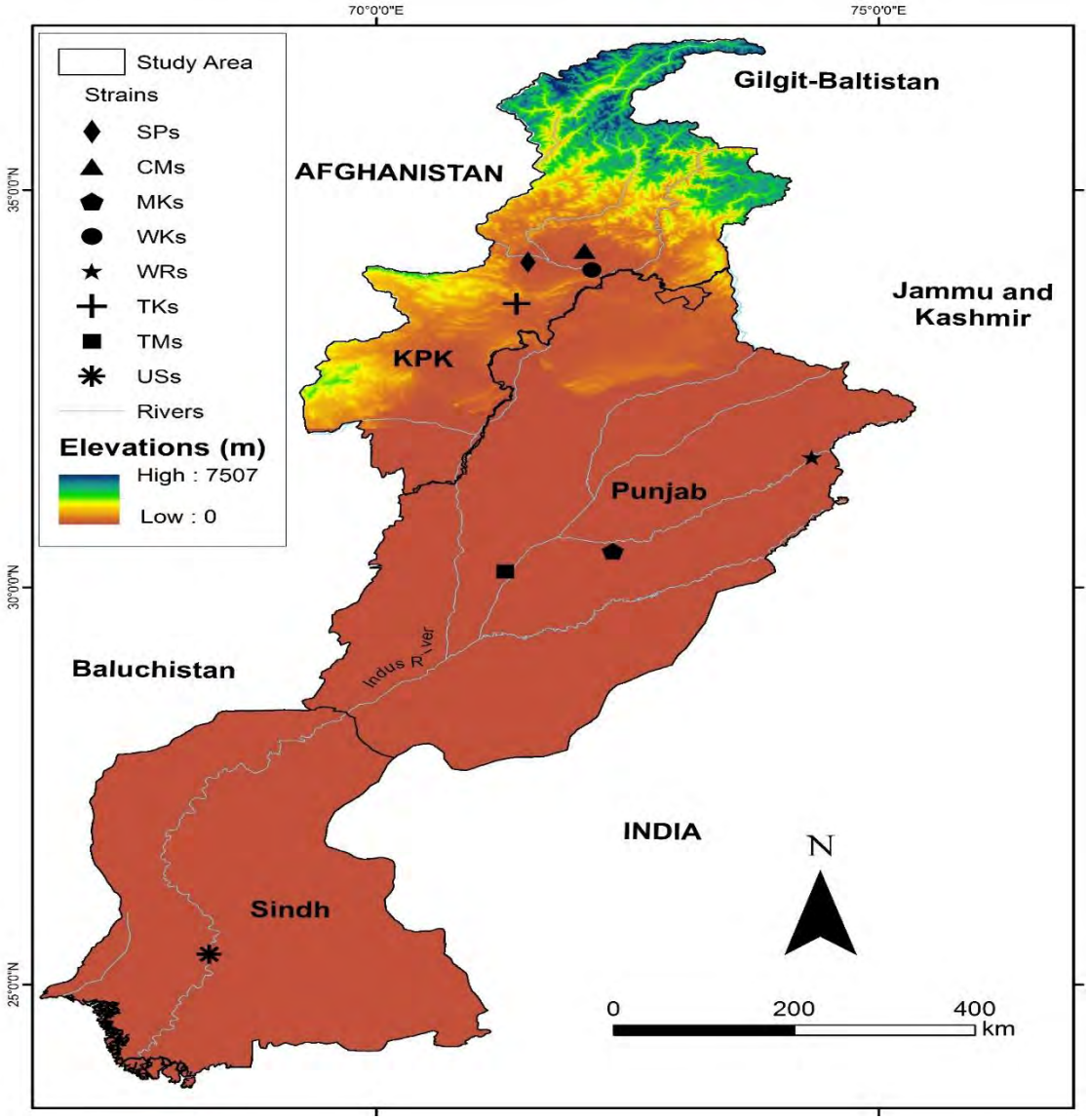


Fig 1: Collection sites of strains used in the study

1. Upper Sindh Fish Hatchery

Sindh has a generally arid and hot environment, with high summer temperatures and mild winters. The monsoon season having rain extends from June through September, has an impact on the region.

The Upper Sindh Fish Hatchery is a Government Fish hatchery. It is projected to focus on breeding and rearing fish species that are suited to the region's climatic conditions including Indian major carp (*L. rohita, Cirrhinusmrigala* and *L. catla*)and Chinese carp like common carp (*Cyprinus carpio*), silver carp (*Hypophthalmichthys molitrix*), bighead carp (*Hypophthalmichthys nobilis),* andgrass *carp (Ctenopharyngodonidella).* This hatchery has well-developed induced breeding facility and number of

nursery and rearing earthen ponds. Here semi-intensive culture practice is commonly used for culturing fish species.

2. Carp Hatchery and Training Center,

It is in Peshawar, Khyber Pakhtunkhwa, Pakistan. The climate of Peshawar is temperate, with scorching summers and cold winters. The region receives large temperature changes throughout the year, with more rainfall during the monsoon season. The carp hatchery and training center is popular in province for the breeding and rearing of carp species but recently other species such as catfish, and ornamental fish species have also been introduced to the setup. Pond culture, feeding management, water quality monitoring, seed production through induced spawning, seed dispersion to local farmers and for conservation purposes, and disease control are all part of carp farming practices. However, training programs for local fish growers receive special attention.

3. Tawakkal Fish Hatchery

It is in District Muzaffargarh, province of Punjab, Pakistan and was established in 1995. The vast areas of Muzaffargarh are plain and have well-established canal systems to provide water to nearby farms. Muzaffargarh has a hot desert climate with exceptionally high summer temperatures and mild winter. The temperature ranges from 520C in June to 40C in January. The annual average rainfall is 127. In this region scarcity of water is an issue during fish breeding practices due to insufficient rainfall. However, well-aerated tube well water is utilized for fish breeding and rearing at Tawakkal-Hatchery.

Typical cultural practices at Tawakkal Fish Hatchery include the breeding and rearing of warm-water fish species such as tilapia, Indian major carp, and catfish species. This is a private Hatchery, has well-trained staff and uses best management practices for rearing/culturing fish, controlling fish diseases and conserving water. In addition, this hatchery has a huge number of broodstock of each species, a well-developed fish breeding set-up and has many nursery and rearing tanks.

4. River Ravi

The climate around the Ravi River is typically subtropical, with hot summers and somewhat cold winters. Temperature and precipitation vary seasonally in the region.

5. Govt. Fish Hatchery, Mianchannu

It is in Mian Channu, Punjab, Pakistan. Mian Channu's climate is semi-arid, with scorching summers and comparatively cold winters. Rainfall in the region is moderate, especially during the monsoon season.

The Government Fish Hatchery in Mian Channu is focused on producing fish species suitable for the local climate, such as tilapia and Indian major carps (*L. catla, L. rohita, C.mrigala*). In addition, Chinese carps like *H. molitrix, C. carpio, C. idella, and H. nobilis* are also cultured at the hatchery. The hatchery has a mixture of concrete and earthen ponds for the rearing of fish. This hatchery is in the practice of using commonly used Induced breeding, seed production, seed dissemination, feed management, and disease control methods.

6. River Kabul

The climate near the Kabul River varies depending on where you are along the river. It is often classified as subtropical or temperate, with temperature and rainfall patterns varying.

7. Govt. Fish Hatchery, Charbanda, Mardan

It is in Khyber Pakhtunkhwa, Pakistan. The climate of Charbanda, Mardan, is temperate, with hot summers and cold winters. Throughout the year, the region receives moderate to heavy rainfall.The Government Fish Hatchery at Charbanda focuses on breeding fish species suitable for the local climate, such as Indian major carps and Chinese carps. The hatchery has a numberof circular tanks for breeding and earthen ponds for fish production are two common cultural practices while feeding practices, disease management are also employed. To develop aquaculture in the region, the hatchery engages in breeding, seed production, and outreach programs.

8. Tanda Govt. Fish Hatchery Kohat, Province Khyber

It is in Kohat, Khyber Pakhtunkhwa Province, Pakistan. The climate of Kohat is semi-arid, with scorching summers and cool winters. During the monsoon season, the region receives modest rainfall.

The Tanda Govt. Fish Hatchery in Kohat focuses on producing fish species suitable for the local climatic circumstances, such as Indian major carps (*L. catla, L. rohita, C.mrigala*). In addition, Chinese carps like *H. molitrix, C. carpio, C. idella, and H. nobilis*Cultural practices include the utilization of ponds and circular tanks for fish raising, as well as a well-organized concrete pond setup at the hatchery. Their activities include induced breeding, hatchery management, disease control, and fish seed distribution.

DNA Sample Collection

From all locations, twenty fish per population/strain were collected, and their part of the caudal fin was removed, washed with 75% alcohol to remove any impurity, and then carefully placed in Eppendorf tubes containing 90% ethanol and transported to Research Centre in ice box while maintaining 4°C temperature for DNA extraction (Lutz et al., 2023).

Extraction Protocol of DNA

A modified salt extraction method reported by Kumar et al. (2007) was employed to isolate the DNA from the caudal fin of each specimen. Briefly, the fin was cut into small pieces and dried on filter paper. Afterwards, 20 mg of macerated fin was placed in a 5 ml tube containing 1.5 ml lysis buffer (200 mM Tris-HCl (pH 8.0); 250 mM NaCl, 100 mM EDTA, 60 µl of 20% SDS, 10µl Proteinase K (20 mg/ml). The contents were mixed well and incubated at 48°C for 3 hours in a water bath. Then, 800 µL of phenol-chloroform-isoamyl alcohol (25:24:1 ratio) was added to the lysed contents and tubes were shaken manually for 5 min. The mixture was then centrifuged for 10 min at 10,000 rpm, and the supernatant was collected in a tube containing 800 µL of chloroform-isoamyl alcohol (24:1 ratio). The mixture was mixed well manually and again centrifuged, and the supernatant was collected in a new tube. Afterwards, DNA was precipitated using sodium acetate (NaAc, pH 4.8) and isopropanol and dissolved in 35 µL of ultrapure water.

DNA quantification

The quantity and quality of the DNA obtained were evaluated using NanoDrop (ND-1000 Spectrophotometer, NanoDrop Technologies, Inc. USA). NanoDrop works on the principle of UV-visible absorbance. Generally, nucleic acids and purified proteins absorb light at a wavelength of 260 nm and 280 nm respectively. This instrument measures 2µl of DNA within seconds. Thus, the quantity of DNA in a sample was estimated by setting Nanodrop at 260 nm while the ratio of absorbance at 260nm and 280nm was used to determine the purity of DNA. Samples having high quantity and quality of DNA samples were selected for subsequent analysis.

Gel electrophoresis

To confirm the presence of the extracted DNA, electrophoresis was performed using an Agarose Gel Electrophoresis system (ThermoFisher Scientific, USA). Briefly, TAE buffer (50X stock solution) was prepared by mixing 242 g of Tris base and 700 mL of autoclaved water at 37°C. Once the Tris base was completely dissolved, then 100 mL EDTA (0.5M, pH 8.0) and 57.1 mL of glacial acetic acid were added. Subsequently, the solution was adjusted to 1 L with Ultrapure water (Milli-Q water). Afterwards, TAE (1X buffer) was prepared from the 50X stock solution and kept at room temperature for further use.

The 1X TAE buffer was used for the preparation of 1% agarose gel. Briefly, 1 g of agarose was weighed and added to a 250 mL beaker. Then 99 mL of 1X TAE was added and heated in an oven for 2 minutes. Once the agarose dissolved and the solution became transparent, 2 µL of Ethidium bromide dye was added. Subsequently, the mixture was transferred to the gel tray and allowed to cool for 15-20 minutes. Afterwards gel tray was placed into the casting apparatus and the comb was set into the gel mold.

After the solidification of agarose, the combs were removed from the gel caster, and 250 mL of 1X TAE buffer was added to the electrophoretic chamber. The gel caster with the solidified gel was then placed in the chamber. Subsequently, loading dye (0.25% each of xylene cyanoldye and bromphenol blue +30% glycerol) was added to the DNA samples at the rate of 5 µL of loading dye per 3 µL of DNA sample) and samples were loaded into the wells of the gel. The electrodes were connected, the power supply was programmed at 120V, and the gel was run for 30 minutes. Gel Documentation System (GelDoc, Biometra, Germany), was used to visualize the gel. The bands were observed, and the images were saved and appropriately labelled for subsequent use.

PCR (Polymerase chain reaction)

Upon verification of the isolated DNA, PCR was conducted to amplify the designated regions within the samples.

Primer selection for microsatellite markers

The Microsatellite study was conducted with three heterologous microsatellite primersalready used by Patel et al.(2009). The three selected primers are given in Table 1 below.

Table 1: Sequence of 3 selected microsatellite primers withtheir PCR conditions used for the analysis diversity in eight strains of *Labeorohita*.

S.No	Primers	Primer Sequence 5′-3′	TM°C
1	Lr-28	F: TTCACGGACAGATTTGACCCAG R: AGTCTTTTCAGGAGATTAGCAG	58
2	Lr-29	F: ACGTAAAGGTCACAAGCTGAAG R: AGCACGGTGTTTGTGTGCGAG	60
3	Lr-37	F: TGAGATGTTCAGCAGGAGCTC R: GAGCGTCGAGTGGCGTTTC	60

Primer selection for mitochondrial marker

A CO1 gene primer designed and used by Ward et al. (2005)for the identification of 207 fish species and assessment of different stocks of farmed *L. rohita* (Kamran et al., 2023) was selected (Table 1). The sequences (Table 2) were sent to Macrogen (Macrogen, Seoul, South Korea), specializing in Humanizing Genomics, and it manufactured the said primer.

Table 2: Sequence of mitochondrial gene CO1primer with their PCR conditions, GC percentageand PCR product size.

Target gene	Sequence 5′ to 3′	TM °C	GC%	Size (bp)
CO1	F: TCAACCAACCACAAAGACATTGGCAC	46.6	46.5	680
	R: TAGACTTCTGGGTGGCCAAAGAATCA		46.5	

Primer Dilution

The stock solution of each PCR primer was prepared by diluting the company-provided primer solution with the addition of a required amount of injection water based on their concentration. Subsequently, 10 µl from this stock solution and 90 µl of injection water were mixed well. The resulting solution was stored at -20ºC for future use.

Reaction Mixture

For each PCR, a 30 µl reaction mixture was usedin a gradient thermal cycler PCR (BIO-RAD T100™ thermal cycler, Hercules, California). Before use, a brief centrifugation, or short spin, was applied to ensure thorough mixing of all the ingredients. The composition of the reaction mixture is shown in Table 3.

Table 3: The reaction mixture for the polymerase chain reaction

Reagents	**Volume (**µl)
PCR Master Mix	14
Primer (Forward)	1
Primer (Reverse)	1
Injection Water	11
FDNA	3µg/ µl
Reaction volume	**30**

PCR conditions

For amplifying Lr-28, Lr-29, and Lr-37 loci, PCR conditions used included 1) a 5-minute initial denaturation, ii) Denaturation at 95 °C for 1 minute, iii) Annealing of Lr-28 at 58 °C, Lr-29 and Lr-37 at 60 °C for 45 seconds, iv) Elongation at 72 °C for 1 minute and then final elongation at 72 °C for 10 minutes. A total of 30 cycles were run in the PCR procedure. Finally, the PCR end products were stored at temperature (4°C) for subsequent analysis. Furthermore, PCR conditions for amplification of CO1 gene included, 1) An initial denaturation at 94 °C for 1 minute, 2) Denaturation at 94°C for 60 seconds, 3) Annealing at 54 °C for 45 seconds, iv) Elongation at 72 °C for 60 seconds, succeeded by 7 minutes at 72 °C. There were 35 cycles for denaturation, annealing, and extension steps.

Validation of PCR results

To analyze the amplified products, and verified the specificity of the primer, gel electrophoresis was conducted by loading DNA ladder (100BP) in one well of each comb, while 5µl of the PCR productsamples containing 1 µl bromophenol blue dye were loaded in the rest of the wells of the 1.5% agarose gel. The gel was run at 180V for 15 minutes.Subsequently, the gel was visualized using a gel documentation system (GelDoc, Biometra, Germany). The bands were observed, and the images were saved and appropriately labelled for subsequent use.

Sequencing

PCR products showing distinct and high-quality bands were sent to BGI genomics company (Hong Kong) for sequencing. The sequencing procedure was done utilizing both the forward and reverse primers, adhering to Sanger's method (Sanger et al., 1977).

Sequence analysis by bioinformatics tools

The acquired sequences were transformed into FASTA format using BioEdit software, and only the sequences with distinct peaks were chosen. Subsequently, these selected sequences underwent BLAST analysis on the NCBI GenBank portal (https://blast.ncbi.nlm.nih.gov/Blast) and BOLD (https://www.boldsystems.org) to verify that the acquired sequences are corresponding to the *L. rohita.*

Furthermore, to recognize the regions of similarity, sequence alignment was conducted using BioEdit software (Thompson et al., 1997).

Analysis of microsatellite data

Manual scoring of alleles, represented by bands on the microsatellite loci, was undertaken. Band sizes were computed using the DNA fragment program (version 3.03), following the methodology outlined by Nash (1991). The identification of specific alleles at each locus was accomplished through genotype scoring, as illustrated in Figures 2, 3, and 4.

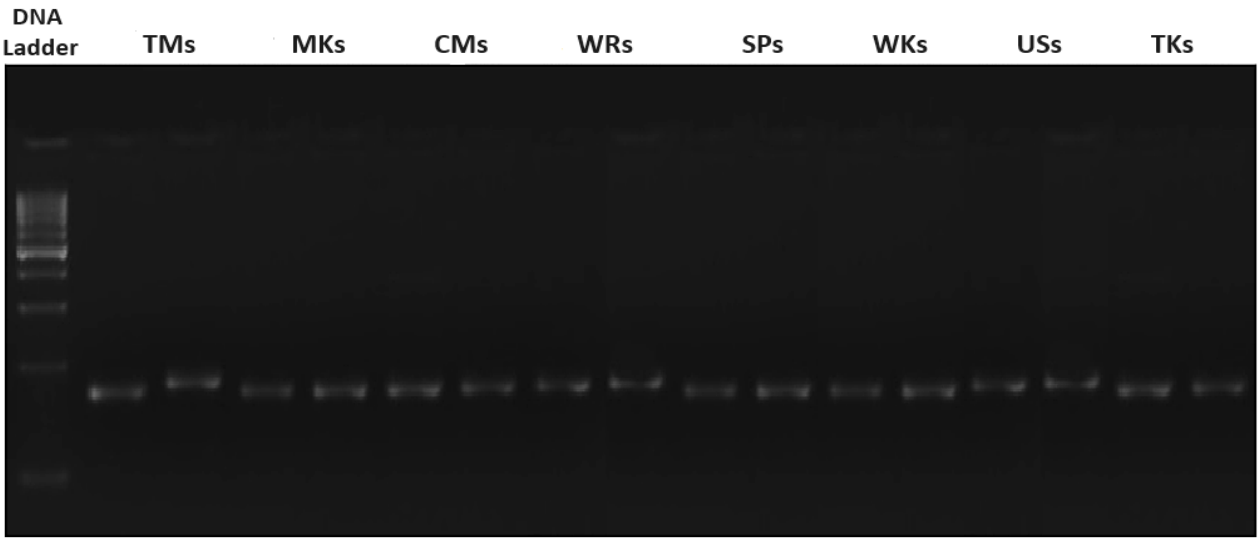


Fig 2: Multiple bands of eight different *Labeorohita* populations, amplified by microsatellite marker Lr-28.

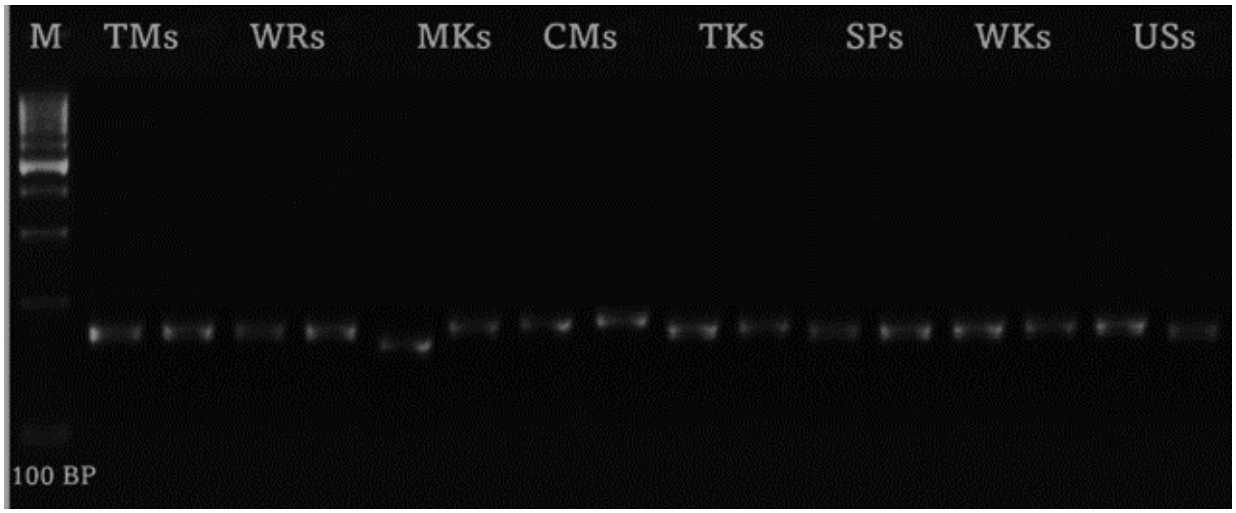


Fig 3: Multiple bands of eight different *Labeo rohita* populations, amplified by microsatelite marker Lr-29.

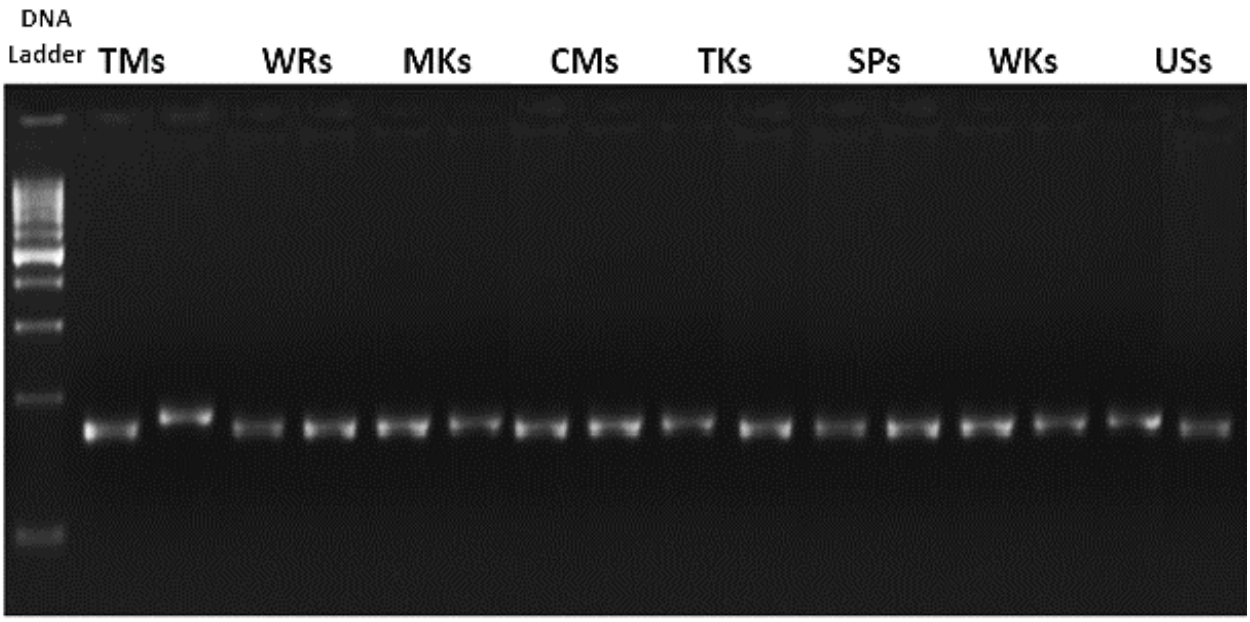


Fig 4: Multiple bands of eight different *Labeo rohita* populations, amplified by microsatelite marker Lr-37.

Analysis of COI gene

Afterthe sequenceediting and alignment, Kimura 2-parameter (K2P) model (Kimura, 1980) was used to determine the extent of sequence divergence among various strains. Phylogenetic analyses were conducted through the implementation of the neighbor-joining and maximum likelihood methods, using Molecular Evolutionary Genetics Analysis version 7 (MEGA, Englewood, NJ).

Analysis of molecular variance (AMOVA)

AMOVA was done using the DnaSP5.exe software and comprehensively investigated the spatial structure of strains. This analytical approach was applied to both microsatellite and mitochondrial markers, allowing for a thorough exploration of genetic variability within and between populations. The assessment involved computing population pairwise FST statistics, which measure the genetic differentiation among populations. Significance levels were determined at a threshold of $p = 0.05$, ensuring a robust evaluation of the observed genetic variations. This comprehensive analysis aimed to provide insights into the population dynamics and genetic structure of the selected strains.

Experiment 2. Comparative growth performance and innate immunity of Frys, and fingerlings of different strains of *Labeorohita*

In the second phase of the research, Frys, and fingerlings of different strainsof *Labeorohita*were reared under controlled and semi-intensive culture system respectively at Fisheries and aquaculture researchfacility (FARF), Quaid-i-Azam university and their comparative growth performance was evaluated.

For the collection of hatchlings of each strain, fish breeding experiments were conducted (during June to early August) at the selected Hatcheries. For wild strains, adult fish (about 3 years old) were collected during mid-September to October from River Ravi and River Kabul with the help of fishermen and transported to Quaid-i-Azam and stocked in well fertilized earthen ponds at FARF. After two days of shifting, fish were provided 32% crude protein floating pellet feed (Aqua Feeds, PVT LTD) at the rate of 2% body weight (BW). The feeding rate gradually decreased with fall in temperature and increased with an increase in water temperature. During April to June fish were fed at the same ration (2% BW) daily. Fish were maintained in ponds for about 7 months before the initiation of breeding experiment at FARF, QAU. During growing season, the water quality parameters likedissolved oxygen (DO level)

temperature, and pH were checked on daily basis and kept under optimum range, while fertility of ponds was checked with a Sacchi disc and maintained with the addition of organic (Animal manure) and inorganic fertilizers (Diammonium phosphate (DAP) or urea). Fresh and aerated tube well water was added daily at dawn and dusk to control the oxygen level. At the time of stocking, the water temperature was 22°C which fell to 18°C during end of January and againincreased and reached 28°C in June.

Fish Breeding experiment

Fish breeding experiments at each Fish Hatchery were conducted during June to early August by using broodfish of about 3 to 3.5 years old. Briefly, 3 to 4ripen dams and 5-6 sires*L. rohita* broodfish were harvested from the earthen ponds and their ripeness was checked by observing secondary sexual characteristics reported by Metwally *et al*. (2008), like ripen female has soft/ distend belly while in male white color milt is emitted with slight pressure on the abdomen. The ripenedbrood fish were transported to hatchery building and kept in holding tanks having well aerated water.

Induced Spawning

Induced breeding of *L. rohita* at each hatchery and wild was conducted by following the procedure reported by Ahmad et al. (2019). Briefly, after 3-4 hours of conditioning in holding tanks, male and female brood fish were weighed individually and injected Ovaprim (LHRH analogue + dopamine antagonists, Syndel, USA) intramuscularly at the rate of 0.5 ml/ kg to both female *L. rohita* and 0.2 ml/ kg body weight to male broodfish (Ahmad et al., 2019). After injecting the induced spawning agent, fish were kept in a circular tank (2 dams and 3 sires/tank) with slow-moving water. The disturbance was avoided by covering each circular tank with a canvas screen. About Seven to eight hours after the injection of Ovaprim, female broodfish/dams were constantly observed. When the female released their eggs and the male fertilized them, then both male and female brood fish were removed from the circular tanks. The fertilized eggs were left in circular tanks for incubation and hatching at ambient temperature. The hatching of fertilized eggs was completed within about 19 hours. The newly hatched yolk-sac larvae remained kept for a further 72 hours or till the yolk-sac was absorbed in the incubation tank with continuous slow-moving water. Afterwards, post larvae were stocked in nursery ponds and reared under a semi-intensive culture system.

During the breeding experiments at Kohat Hatchery, broodfish did not spawn, so this strain was excluded for further study and fish from Rawal Hatchery and Research Training Centre, Islamabad, were included in the study to maintain diversity.

Procurement of fry

Healthy Frys of about 1.0 to1.2 g body weight were collected from each hatchery and transported to FARF, QAU by adopting closed live hauling techniques. They were stocked in fiberglass tanks and reared until reached about 1.5g body weight. Fry of Wild strains (WKs and WRs) of similar weight (about 1.2 g BW) were also transferred from nursery ponds of FARF to fiberglass tanks and reared. During rearing they were provided 32%CP diet twice a day.

Rearing of advanced fry under controlledconditions

An experiment in triplicate under controlled conditions was executed. Frys of each strain when reached approximatelyto 1.5 g BW were transferred to aquaria (size: 60 × 60× 40cm^3) at a stocking density of 1.5g/L (80 fries/aquarium). The aquaria were well equipped with aquarium heater (XL-999 500W) and air stones connected to Air pump (HAP-120, Hailea, China) to maintain temperature 27 °C and dissolved Oxygen 6.0 mg/L.Moreover, 14:10 hoursphotoperiod was set. After the shift, the advanced fry was acclimatized to experimental conditions for about three days.

Feeding strategy

Initially, 40 % CP crumbled feed (Aqua Feed, PVT LTD) was administered four times daily (8:00 hours, 12:00 hours, 14:00 hours, and 16:00 hours) at 6% body weight. After two weeks, at random fish from each aquarium were collected, weighed, and adjustments to feed particle size and frequency were made. Additionally, 2 hours after feeding, unconsumed feed was collected, dried, and stored for the determination of feed conversion ratio (FCR) through the following equation:

Feed conversion ratio (FCR) = feed intake (g) ÷ wet weight gain (g)

Water Quality

Throughout the rearing process, water temperature (°C), DO (mg/L), and pH were noted twice daily at 09:00 hours and 16:00 hours) using a Multiparameter device (Model HI-9828, HANNA Instruments, Inc., Woonsocket, USA). The total ammonia level of each aquarium was checked weekly with the help of an ammonia test kit (HI3824, ROMANIA). Through the rearing period, i.e., 30 days, DO levels, and pH in all aquaria exhibited minimal fluctuations (±0.3°C), while the ammonia concentration consistently remained below 0.035 ppm.

Growth performance

Following a30-day experiment under controlled conditions, the fish were subjected to a one-day fasting period. On the sampling day, fingerlingsof each strain from their respective aquaria were collected, and weighedcollectively, then counted the total number, calculated individual average weight and determined the growth performance metrics,by using following standard formulas.

Weight gain = FBW– IBW

$$WG\ (\%) = \frac{(\text{FBW of the fingerlings} - \text{IBW of the advancedFrys})}{(\text{IBW of Frys})} \times 100$$

$$SGR\ (\%) = \frac{ln\ (\text{FBW}) - ln\ (\text{IBW})}{\text{Rearing period (days)}} \times 100$$

$$\text{Survival rate}\ (\%) = \frac{(\text{Number of Fingerlings received} - \text{Number of Frys stocked})}{\text{Number of Frysstocked}}$$

WG: - Weight gain

FBW: - Final body weight

IBW: -Initial body weight

SGR: - Specific growth rate

Sampling for hematological and immunological indices

Fingerlings underwent a 24-hours fasting period before collection of blood. Following rearing for 30 days, five fish were captured from each aquarium using a hand net. They were then subjected to anesthesia using AQUI-S (0.5 mL/100 mL), and blood was extracted from the fish through tail ablation. This method was chosen as the fish were unsuitable for blood extraction using a syringe.As the blood from a single fish proved insufficient for analysis, the blood from five fish from each aquarium was collected in a K2 EDTA tube (BD Vacutainer®), forming one composite sample.

For the evaluation of immunological parameters, blood sampling was done by the same procedure, i.e., 15 fingerlings/aquarium, blood of 5 fish was pooled (thus 3 samples/ aquarium or 9 samples / strain). Blood was left undisturbed at room temperature for about 70 minutes and allowed to clot. Following the removal of the blood clot, serum separation was achieved by centrifugation at 2000 rpm for 15 minutes. The upper layer, i.e., serum was collected in a tube and saved at low temperature (4°C) till further analysis. For the analysis of the respiratory burst activity and phagocytic activity, fresh blood was used.

Growth performance of fingerlings

For raising fingerlings of each strain to a reasonable and almost similar size for PIT tagging, thinning and manipulation of stocking density were performed. Fingerlings of CMs, IBDs and SPs were stocked at the rate of 1.5g/L (35 fingerlings/tank), USs at 1.7g/L and others at 2g/L. They were reared under optimum conditions (temperature, 27.5 ±0.5°C; DO, 5.6±0.5) until body weight reached above 40g. During rearing, all strains were provided locally available commercially prepared feed (Aqua feed, Pvt Ltd; pellet size: Crumble to 2 mm pellet, crude protein: 35 to 38%) twice a day and allowed to be fed ad libitum. Furthermore, to minimize body weight differences among different strains, manipulation of stocking density by changing the water level and the number of fish in a tank was practiced.

Tagging

When fingerlings of each strain reached above 40 g body weight, then to distinguish them, making was done with Passive Integrated Transponder (PIT) tags. Prior to tagging, the fish underwent a one-day fasting period, and for tagging, fish were anaesthetized with buffered Tricaine methane sulfonate (MS222; 50mg/L) solution. The implanter was sterilized with 70% alcohol and BIO8. B.03V1 HPT8

MiniChip tags were scanned before insertion. The PIT tags were inserted with the help of a sterile implanter into the peritoneal cavity of the fish after creating sufficient space. After insertion, KMNO4 was applied to the implantation site for disinfection, and tagged fish were released in well-aerated water-holding tanks to recover from stress. Fish were allowed to remain there for about a week. Once successfully recovered, tagged fish were stocked into well-prepared earthen ponds.

Rearing of fingerlings under a semi-intensive culture system

Preparation of Earthen ponds

The rearing of tagged fingerlings was done in rectangular ponds (average area of each,250 m^2 and a depth of 2.0 m) at FARF, QAU. Prior to stocking of the fingerlings of various strains into the earthen ponds, three adjacent ponds underwent sun-drying, and damaged dikes were repaired. By adopting methods for the preparation of earthen ponds before stocking (Sayeed et al., 2007; Ullah et al., 2018), calcium carbonate (125 kg/ha) was evenly spread,and ponds were fertilized with animal manure (cow dung; 333.33 kg/ha). The animal manure was distributed evenly across the pond's bottom andallowed to dry in sunlight for several days. Subsequently, the ponds were initially partially filled with water. When the water became fertile, additional water was introduced, filling the ponds up to 1.5 m. Pond productivity and water levels were maintained through the addition of fertilizers (animal manure and Diammonium phosphate (DAP)) and water. Periodically, the productivity of the ponds was checked using Sacchi disc, and a reading below 30 cm indicated the restriction of fertilizers.

Experimentaldesign

To get the true ranking of each strain with respect to growth performance, acompletely randomized growth trial in triplicate was designed and conducted in communal ponds under semi-intensive culture system by adopting previously reported method (Kamran et al., 2023). Briefly, 75 PIT tagged F1 fish of each strain having no sign of disease were evenly distributed in three earthen ponds (200 fingerlings/pond or 25 fingerlings/strain /pond). After three days of shifting, prepared feed was started to be provided.

Feeding strategy

Fish wereprovided with 32% CP locally available pelleted feed (Aquafeed Pvt. Ltd; size, 2mm). Fish fed twice a day (at 8:00 hour and 16:00 hours) at 3% body weight, approximately two-thirds of the calculatedfeed was fed in the morning, while the rest in afternoon. Fortnightly at random 10 to 15fish were captured from a pond, weighed, and feeding ratio and %CP were adjusted with the change of fish weight and size. Initially, the pellet size of 32% CP diet was 2mm but ended with 4mm pellet having 28 % CP.

Water Quality

Throughout the rearing period, pond water quality parameters like temperature, DO level, and pH, were daily noted by using a multiparameter (HI-9828 HANNA Instruments. Inc. Woonsocket, USA). Fortnightly total ammonia was also checked using an ammonia meter (Hanna Instruments HI-715 Checker). At the time of stocking of fingerlings, the water temperature and DO levels in three ponds were 25.5±0.4˚C, and 5.6± 0.5 mg/L. Throughout the rearing phase, fluctuations were observed in water temperature (ranging between 25.5-28.5˚C), DO levels (ranging between 5.4-6.2 mg/L), and pH 7.5±0.5). Conversely, ammonia levels (<0.035 mg/L) exhibited no noticeable changes. In this part of the study, the F1 fingerlings from all strains in each pond shared a similar rearing environment throughout the experimental period.

Sampling

At the time of stocking, the total count and biomass of each fish strain along with the individual weight of each fingerling was noted. Fortnightly, growth was checked by random collection of 6-8 fish from the pond, individually weighed and then feeding was adjusted accordingly. At the endof rearing period under a semi-intensive system in communal ponds, feed was restricted for one daybefore sampling. On the day of sampling, a screen was set on the outlet of each pond and water was drained stepwise. Following the harvest, fish were segregated by strain using a Pit tag reader, collectively weighed andcounted the number for the calculation of the average weight.

Standard formulas as outlined above were employed to assess the survival (%), growth performance (WG, WG %, SGR), and production such as biomass.In this experiment, FCR was not measured,

because of the presence of all strain in the same (communal) pond and impracticality of collecting undigested feed or feces of each strain separately. Moreover, 3 fish/pond/strain were dissected aseptically on ice pad and their liver tissues were sampled, collected in autoclaved Eppendorf tubes containing RNALater™ for the isolation of RNA isolation for studying expression of genes related to growth (Myogenin, MyoD, IGF-11, Preproghrelin), innate immunity (Lysozyme C, Lysozyme G, TNF **α,** Natural Killer enhancing factor(NKEF),and stress (HSP 70 & HSP 90). Moreover, their muscle tissues were saved for proximate analysis.

In addition,blood sampling was also done to compare the hematological and innate immunity indices of the F1 fish of all strains. For these 18 adult fish/strain (6 fish/communal pond) of all strains were collected at random, anesthetized with buffered MS222 (100mg/L) and the blood was drawn from the caudal vein of each fish using a 2cc heparinized syringe and blood of 9 fish (3fish/pond/strain) were collected in VACUETTE® EDTA K3 tubes for hematological indices while blood of other 9 fish /strain were collected in 2 ml Eppendorf tubes for the separation of serum and analysis of immunity parameters.

Evaluation of haematological indices

Blood samples were processed and analyzed employing an automated hematology analyzer (Auto Hematology Analyzer RT-7600), generating a comprehensive blood profile.

Immunological parameters

To separate the serum, the blood samples were spun in a centrifuge at 3000 revolutions per minute (rpm) for 5 minutes. After centrifugation, the serum from each sample was carefully transferred into separate Eppendorf tubes and kept at 4°C for storage. These stored serum samples were designated for future analysis, including analysis of aspartate aminotransferase (AST), total serum proteins, immunoglobulin (IgM), and lysozyme activity. Additionally, fresh heparinized blood was used to assess phagocytic activity and respiratory burst activity.

AST activity

The AST/GOT kit obtained from AMEDA Laborodiagnostik GmbH Graz Austria was employed to analyze AST activity, following the standardized protocol provided with the kit.
In summary, 100 µL of serum was mixed with 1000 µL of working reagents, consisting of 910 µL of reagent A and 91 µL of reagent B, in a 2 mL Eppendorf tube. The resulting mixture was gently stirred and then incubated for 1 minute at 30˚C. Subsequently, a UV-Visible spectrophotometer (Model Micro-spectroAgilant 8453), set at λ 340 nm, was used to record absorbance readings at one-minute intervals, and this process was repeated three times. The quantification of AST activity was determined and expressed in U/L.

Total immunoglobulin and total protein in serum

The procedure described by Lowry et al. (1951) was used to measure the total protein content in blood serum. The standard protein, bovine serum albumin (BSA), was first dissolved in 1 mL of distilled water (w/v) to prepare a stock solution in a test tube containing 1 mg of BSA. Subsequently, various standard solutions with distinct concentrations were prepared by diluting the BSA stock solution (ranging from 0.05 to 1 mg/mL) with distilled water, ensuring each solution reached a volume of 5 mL in a test tube. To analyze the protein concentration, 0.2 mL of the protein solution was transferred into various test tubes, each containing different dilutions. To each test tube, 2000 µL of alkaline copper sulfate reagent was added, and the solutions were thoroughly mixed before being left to incubate for 10 minutes at room temperature. After the incubation period, 200 µL of Folin-Ciocalteau solution was added to each tube, followed by another 30-minute incubation. Subsequently, the absorbance of the solutions was measured at 660 nm using a spectrophotometer. A standard calibration curve, generated using known protein concentrations, was plotted against absorbance. The protein concentration of an unknown sample was then determined by comparing its absorbance to the standard curve.
For the determination of serum immunoglobulin, the approach detailed by Anderson and Siwicki (1995) was adopted. In summary, 100 µL of serum was mixed with 100 µL of polyethylene glycol (12%), leading to the separation of immunoglobulin from the serum through precipitation.

The solution was shaken at room temperature for 120 minutes on an Incubator shaker (ISS Innova 43), then centrifuged at 7000 × g for 10 minutes. The absorbance of protein concentration in supernatant and

serum was measured at 660 nm separately. To calculate Total IgM, subtract the protein amount in the supernatant from the total protein concentration in the serum.

Lysozyme activity

To measure the activity of lysozyme, 900 µL of Micrococcus lysodeikticus (Sigma, St. Louis, MO, USA) suspended in phosphate-buffered saline solution (pH 6.2) at a concentration of 750 µg/mL was mixed with 100 µL of serum. After the mixture was well combined, a UV-visible spectrophotometer (Model Micro-spectroAgilant 8453) was used to track the rate of absorbance change at 450 nm for ten minutes, at 1-minute intervals. White lysozyme from hen eggs was used as a reference to measure the lysozyme activity (Sigma-Aldrich).

Respiratory burst activity

The Nitro blue tetrazolium (NBT) dye method, as reported by Anderson and Siwicki (1995), was used to assess the level of free oxygen radicals in the blood. A mixture of 100 µl blood and 0.1 ml of 0.2% NBT solution was placed in a 2 ml Eppendorf tube. After that, the mixture was allowed to sit at room temperature for half an hour. After the incubation period, 1000 µl of N, N dimethyl form amidewas added to a test tube along with 50 µl of the NBT-blood cell suspension, which was pipetted. This mixture was centrifuged for five minutes at 3000g. The supernatant was then moved to a glass cuvette so that a UV-visible spectrophotometer could measure the absorbance at 540 nm. The same materials were utilized for the blank control; however blood was replaced with distilled water, and the absorbance at the same wavelength was recorded.

Phagocytic activity

To evaluate the phagocytic activity of phagocytic cells, the methodology outlined by Anderson and Siwicki (1995) was followed, employing *Staphylococcus aureus* (Sigma, St Louis, MO, USA). Briefly, 0.1 mL of heparinized blood was added to each well of a microwell plate, along with 100µL of killed *Staphylococcus aureus* (1 × 107 cells) and 100µL of saline phosphate buffer (pH 7.2). The mixture underwent a 30-minute incubation at room temperature with intermittent mixing. Next, a 5µL sample was pipetted onto a sanitized glass slide, spread, allowed to air dry, fixed for five minutes in 95%

ethanol, and then allowed to air dry once more. For every sample, two smears were made. Giemsa stain (7%) was applied to the smears and left on for 10 minutes. Next, 100 phagocytic cells were counted from each smear and examined under an Optico XSZ-107T light microscope to determine the quantity of bacteria that were phagocytosed.

A microscope was used to examine 100 phagocytes per slide in order to estimate the phagocytic index (PI) and phagocytic activity. The mean values were calculated as follows: PA = (Number of phagocytic cells with ingested bacteria) / (Number of phagocytes) × 100. PI = (Number of ingested bacteria) / (Number of phagocytic cells).

Antioxidant enzymes assay

To assess antioxidant enzymes in both muscle and gut tissues, homogenization was conducted using a Dounce manual homogenizer (Sigma, Aldrich) in a potassium phosphate buffer solution of 100 mmol concentration, supplemented with 1 mmol of EDTA. Following homogenization, centrifugation was performed at 12,000×g for 30 minutes at 4 degrees Celsius, yielding a supernatant. This supernatant was carefully collected, and various aliquots were prepared in Eppendorf tubes, subsequently stored at -20 degrees Celsius until further analysis of antioxidant enzymes.

Superoxide Dismutase (SOD) Assay

The modified Kakkaret al. (1984) approach was utilised to measure SOD activity. The reaction mixture included 0.3 mL of supernatant, 1.2 mL of 0.052 mM sodium pyrophosphate buffer (pH = 7.0), and 0.1 mL of 186 µM phenazine methosulphate solution. 0.2 mL of 780 µM NADH+ solution was added, followed by 1 mL of glacial acetic acid to terminate the reaction after 1 minute. The chromogen generated as a result was measured with a spectrophotometer set to lmba560 nm absorbance wavelength. SOD's one unit is the number of enzymes/mg protein that participate in the quercetin oxidation reaction with 50% maximum inhibition. SOD activity is measured using a molar coefficient of 6.22×103/M cm and expressed in µmoles/min/mg protein.

Catalase (CAT) Assay

Catalase specific activity was determined using the Chance and Maehly method (1955). To mix, add 0.1 ml (100 µl) of supernatant, 2.5 ml of 50 mM phosphate buffer (PBS; pH=5.0), and 0.4 ml (400 µl) of 5.9 mM hydrogen peroxide (H2O2). The absorbance of the reaction mixture was measured at 1-minute intervals using a spectrophotometer calibrated to 240 nm. The CAT result was expressed as nmol/min/mg protein, with 46/M cm as the molar coefficient.

Peroxidase (POD) Assay

Chance and Maehly's (1955) approach, as followed by Bibi (2012), was utilised to determine POD activity. In summary, 2.5 ml of 50 mM PBS (pH 5.0), 0.3 ml of 40 mM H_2O_2, 0.1 ml of 20 mM Guaiacol, and 0.1 ml of supernatant were combined for the reaction. A UV/Visible spectrophotometer measured absorbance at 470 nm after a one-minute delay. POD activity was measured as nmol min-1mg-1 protein, with a molar coefficient of 2.66×104/M cm.

Proximate composition

To investigate the muscle composition, 9 fingerlings were chosen from each strain, with 3 individuals picked from each pond. The analysis took place at the Poultry Research Institute (PRI), adhering to established protocols (AOAC, 2000). The assessment of crude protein and total fat content was conducted using the micro Kjeldahl method and the Soxhlet apparatus, respectively.

Moisture percentage

To determine the moisture percentage, for 48 hours, fish samples were dried in an oven set at 65±3˚C. The following formula was used to get the moisture percentage.

described by (AOAC, 2000):

$$Dry\ matter = \frac{Weight\ of\ sample\ after\ drying}{Weight\ of\ sample\ before\ drying} \times 100$$

$$Moisture\ content = 100 - dry\ matter\ \%$$

$$Moisture\ (\%) = \frac{Weight\ loses}{Original\ weight\ of\ sample} \times 100$$

Crude protein %

The percentage of crude protein in the muscle samples was estimated by using the Micro Kjeldahl method. The nitrogen content obtained from the Micro Kjeldahl method was converted into crude protein using the formula used by Njinkoue et al. (2016). The equation for crude protein analysis is as follow:

Crude Protein (%) = (Total Nitrogen (%) ×6.25 / (Sample Weight (g)) × 100

Fat percentage

Njinkoue et al. (2016) provided a method for determining the crude fat content of the samples. The following equation was used to analyze the fat content:

Fat (%) =(Extracted Fat (g)×100 / Sample Weight (g))

Ash percentage

The ash percentage was calculated after the combustion of samples at 550˚C for one day. The percentage of crude ash content was then calculated using the formula given by **AOAC (2000)**.

$$Crude\ ash\ \% = \frac{Weight\ of ash}{Weight\ of\ sample} \times 100$$

Gene expression

To assess the expression of genes associated with stress, growth, and immunity, a total of 18 fish per strain were analyzed. This included advanced fry (6 fish per aquarium/strain) and fingerlings (6 fish per pond/strain). The selected genes for examination were HSP 70 and HSP 90 (stress-related),

Preproghrelin, Myogenin, and MyoD (growth-related), as well as Lysozyme C, Lysozyme G, NKEF, and TNF α (immunity-related), as detailed in Table 1.5. Tissues from the liver, muscle, and intestine were collected from the dissected fish. To ensure an adequate sample size, 2 fish from the same strain and aquarium/pond were combined, resulting in 9 samples per strain. Subsequently, these samples were stored in RNALater™ and stored at -80°C for further analysis.

Primer designing

For the quantitative gene analysis, primers for both reference and target genes (Table 4) were designed using Oligo Explorer 1.1.2 software. From the NCBI database (www.ncbi.nlm.nih.gov), the mRNA sequences and partial coding sequences (CDS) of the pertinent genes were obtained. Humanizing Genomics at Macrogen then produced the designed primers.

Table No 4:Target genes and sequences of primers

Target Genes	**Primer sequence 5' to 3'**		**TM °C.**	**GC%**	**Accession No.**
Myogenin	F	AGGCGGCGATAACTTCTTCC	62	55	KR:560074.1
	R	TCCGTTTGACAGCAACCTTC	62	50	
MyoD	F	GCTTTCGAGACCCTCAAGAG	61	55	KC:344537.1
	R	RAATCCATCATGCCATCAGAG	61	45	
IGF – II	R	TGGCTTGCTCCTCATCTTG	62	50	KX455871.1
	F	ATCCCAGGAGCGTCTCTTG	62	45.5	
Preproghrelin	R	CGACCCTGTGGTTTCTGAG	62	50	LC271259.1
	F	CCAGCCACTTGTTTGTGC	62	45.5	
Lysozyme C	F	TCCCTGTTCAGATTTGCTTCA	62	42.9	JQ230329.1
	R	TGACAGCTTACGCCCATTAC	62	50	
Lysozyme G	F	GTGGTCCCAAGAGCAATGT	62	52.6	KT 184686.1
	R	GTAGTCTTGTCCTGTGGTTCTG	62	50	
TNF α	F	GTAGTGGACCGAGAGATCATCA	61	50	FN543477.1
	R	TCCTGAGGCATGTCAGTCTT	61	50	

Natural Killer enhancing Factor (NKEF)	F	GCCTCCACCGACTCTCATTT	59	55	KC986940.1
	R	CGTAATCGCGGGAGATGGAT	59	55	
β-Actin	F	ATGAAGATCCTGACCGAGAGA	62	47.6	EU 184877.1
	R	CTCGAAGTCAAGAGCCACATAG	62	50	

RNA Isolation

For the isolation of total RNA from liver and muscle tissues, TRIzol® reagent (15596) was employed. Each extraction utilized approximately 50 mg of tissue, and the TRIzol® reagent was stored at 4°C to prevent any incubation. The RNA extraction followed the subsequent protocol.

RNA extraction and processing

The tissue samples were taken from RNALater™ and placed on ice for 2 minutes. About 50 mg of tissue was homogenized using a pestle in a microcentrifuge tube, and 0.5 mL of ice-cold TRIzol® reagent was added. The homogenate was incubated at 25°C for 7 minutes. Subsequently, 100µL of chilled chloroform was added, and the mixture was vigorously agitated for 20 seconds. After a 5-minute incubation at 26°C, the sample was centrifugation at 11,000 rpm at 4°C for 10 minutes, three distinct layers appeared. The upper layer, a clear aqueous phase containing RNA (approximately 80% of the total RNA). The intermediate layer was a white hazy phase with DNA, whereas the bottom layer was a red phenol phase with protein. The upper, colorless RNA layer was carefully transferred to a newly labeled Eppendorf tube, avoiding the interphase and bottom phase.

To precipitate RNA, 250µl of chilled Isopropanol was added to the separated RNA layer and vortexed for 10 seconds. The mixture was then incubated at 26˚C for 10 minutes. The material was then centrifuged at 11,000 rpm for 5 minutes at 4˚C, forming a pellet. The liquid phase was disposed without affecting the pellet.The RNA pellet was washed with cold ethanol by adding approximately 400µl of DEPC-treated chilled ethanol into Eppendorf tubes containing the pellet. The sample underwent a brief vortexing and was then maintained at -20°C for 10 minutes to facilitate precipitation. Subsequently, centrifugation was carried out at 8000 revolutions per minute (rpm) for 30 seconds at 4°C. Following centrifugation, the RNA pellet was washed, and then air-dried for 5 minutes. It was ensured that the

pellet was not completely dried to prevent loss of stability.The RNA pellet was dissolved in 50µl of RNAse free water and stored at -80˚C until cDNA construction.

RNA quantification and cDNA synthesis

Quantification and assessment of the purity of the extracted RNA were performed using NanoDrop-ND1000. Reverse transcription of 1µg of total RNA was used to generate cDNA using the Revert AidFirst Strand cDNA synthesis kit (Thermo Fisher Scientific, Lithuania). For that purpose, first 10 µl MMX was prepared which contains 2µL of 5X Buffer solution, 1µl of each of following; RevertAid reverse transcriptase, dNTPs and Ribonuclease inhibitor, 2.5µl of Injection water and 2.5µl of Random hexamer primer. Then, 10µl of RNA was prepared by dissolving 2µg/µl of RNA (2µl form RNA having concentration of 500ng/µL) in suitable amount of DEPC water. Subsequently, MMX and diluted RNA solution were mixed and added in to the PCR tube and were kept in PCR Machine (Mastercycler, Gradient Eppendorf, USA). Incubation took place for 5 min at 25 °C, followed by 42 °C for 60 minutes and 95 °C for 3 minutesfor1 hour at 37˚C. cDNA was constructed successfully. cDNA was stored at -20˚C for subsequent molecular studies.

Efficiency ofqPCR primer

PCR efficiencies were estimated by analyzing serial dilutions of cDNA, and the efficiencies were found to be close to 100%. Thus, to evaluate the efficacy of the primer pairs for each gene, duplicate PCR runs were executed, and a standard curve was generated through a dilution series. Three dilutions (1/10, 1/100, and 1/1000) were prepared by combining cDNA from all samples, and each dilution underwent triplicate analysis. The validation of the primers was assessed by computing the intra-assay coefficient of variance, involving the comparison of repeated experiments. Primer efficiency was calculated using the equation described by Radonic et al. (2004): %E = 10(1/SLOPE) - 1 x 100.

Quantitative PCR (qPCR):

To ascertain the variance in gene expression of the target gene across different fish strains, quantitative PCR (qPCR) was employed. The PCR reaction mixture, with a total volume of 10µL, comprised 0.5µL of cDNA, 0.25µL of each primer, 5 µL of SYBR Green (real-time PCR MMX (2×)), and 4µL of injection water. Negative controls were included, substituting cDNA with water (Table 5). The cycling conditions for RT-qPCR involved initial denaturation at 94 ◦C for 300 s, followed by 40 cycles of amplification and quantification (10 s at 94°C, 30 seconds at the primer-specific annealing temperature, specified in Table 1.5), a pre-melting hold at 94°C for 10 seconds, and subsequent high-resolution melting at 90°C for 60 seconds, followed by 15 seconds, with a heating rate of 0.05 per second.Each sample's mean threshold (Ct) value, which represents the cycle number at which the fluorescence intensity rises noticeably above the background level, was used for analysis. For each sample, beta-actin was used as a normalizer and positive control for mRNA. The MyGo 3.4.8 real-time qPCR apparatus was utilised throughout the whole expression investigation.

Table 5: The reaction mixture of Real-time polymerase chain reaction

Reagent	**Amount**
cDNA	0.5µl
Reverse primer	0.25µl
Forward primer	0.25µl
SYBER green MMX (2X)	5µl
Water	4µl
Total	**10µl**

Analysis of Gene Expression

The expression level of each gene was assessed using the relative quantification method, as mentioned byPfaffl et al. (2001).

Experiment 3. Comparative effect of acclimated temperatures on thermal tolerance, and

expression of HSP genes in different strains of *Labeorohita*

The experiment was conducted in two phases. In the first phase, fingerlings of all strains were acclimated at different temperatures and then their thermal tolerance was evaluated. In the second phase of experiment, fingerlings of all strains after acclimation at 26±1^{o}C were subjected to low (13°C) and high (37°C) thermal stress and then comparative expression of HSP genes were analyzed.

Fish acclimation

Fingerling of all strains (60 total for each strain), average body weight 22 to 25 g were collected from earthen ponds and transferred to the holding tank having flow through water. For external identification, each strain was marked on the dorsal fin with waterproof tags of different colors and released back in the holding tank to recover from marking stress. For a week prior shifting of fish to aquaria for initiating experiment, fingerlings of all strains were provided 35% CP commercial diet twice a day and allowed them to feed at *ad libitum.*

Experimental design

Thermal tolerance

To assess the thermal tolerance of each strain, we followed the methodology outlined by Ahmed et al. (2022), employing a factorial design that included eight strains (20 fish per aquarium and 60 fish per strain) and four acclimated temperatures. Due to facility constraints, we conducted this set of experiments twice, initially involving four strains, followed by another set of four strains. The acclimatized fingerlings of four strains were transferred from the water holding tank to experimental aquaria (140 × 65 × 65 cm3, ∼590L). These aquaria were well equipped with air stones connected to an air pump (HAP-120, HAILEA) to keepDO levels at 5.6 ±0.2^{o}C mg/L. Additional equipment included 300 W microcomputer temperature controller immersible water heaters (Jiayu MI-300 W) and a locally made 1/4 HP Tank Chiller were also used to regulate water temperature at the desired degree Celsius.

To mitigate the impact of extrinsic factors, 6 F1 fingerlings of each 4 strains were housed in the same glass aquarium (24 F1 fingerlings/aquarium). A thermal tolerance test was performed in a replicate of three, resulting in six aquaria (three each for critical thermal maxima (CTmax), and critical thermal minima (CTmin) respectively) for every strain (18 F1 fingerlings/strain/CTL) for each acclimated temperature. To maintain the selected acclimated temperature, a procedure previously reported (Syed et al., 2018; Ahmed et al., 2022) was adopted, and the water temperature of each aquarium was gradually increased or decreased at a rate of 1ºC/day till reached the target acclimation temperatures (22, 26, 30, 34ºC). Once the desired temperature was reached, the fingerlings were maintained at that specific acclimated temperature for 30 days before the commencement of the trial. The selection of acclimation temperatures was based on literature related to *L. rohita* (Chatterjee et al., 2004; Das et al., 2004; Syed et al., 2018; Ahmed et al., 2022). Throughout the experiment, water quality was maintained by regularly observing water temperature and dissolved oxygen (DO) concentration and exchanging 20-25% of the water of each aquarium with freshwater after 2 to 3 days. After the 30-day acclimation period at the chosen temperature, feeding was restricted for a day, and the thermal tolerance assay was conducted.

Thermal tolerance estimation

Critical thermal methodology (CTM) described by many researchers (Mora and Ospina, 2001; Das et al., 2004; Syed et al., 2018; Ahmed et al., 2022) was adopted for the evaluation of strain-based thermal tolerance. This method is more effective as the endpoint of this assay is disorganization in the movement of the fish instead of death. Thus, this methodology can be used for threatened and endangered species (Bennett and Beitinger, 1997). Both CTMax and CTMin tests were carried out by gradual increase or decrease of water temperature at the rate of 0.3°C per minute of each aquarium set at acclimated temperature till fish displayed loss of equilibrium (LOE), i.e., unable to maintain dorsoventral orientation for ~ 1 minute (Eme and Bennett, 2009). The highest and the lowest temperature at which fingerlings of a particular strain showed LOE were observed and considered as CTmax, and CTmin of that strain, respectively. During the experiment, proper aeration was maintained, while water temperature fluctuations were recorded with a waterproof thermometer attached to the inside wall of each experimental aquarium. The upper and lower thermal limit of every strain was determined by recording the thermal tolerance of every individual within the replicate group. Subsequently, the grand mean was calculated by computing the mean of replicate and considered as the CTmax and CTmin of

the strains. Once the thermal tolerance assay was completed, the tested fish were transferred to water holding tanks having well-aerated water, allowing them to recover successfully.

Thermal stress and expression of HSP genes

To investigate the effect of low and high temperature stress on the expression of heat shock protein genes, healthy fingerlings of each strain were acclimated to 26±1^{o}C and subjected to low (13°C) and high (37°C) thermal stress. The low and high thermal ranges were chosen based on the thermal tolerance assay, where CTMax and CTMin showed loss of equilibrium at approximately 37°C and 13°C, respectively. The temperature of the experimental tank gradually increased or decreased at a rate of 0.30°C per minute. Before and after exposure to respective temperature, the fish were dissected, and liver samples were taken from each strain and stored in RNALater^{TM} at -20°C until RNA extractionand evaluation of comparative expression of HSP70 and HSP90 genes.

Analysis of mRNA levels of HSP genes

Primer designing

For the comparative quantitative expression of HSP genes, primers for both reference and target genes (Table 6) were designed using Oligo Explorer 1.1.2 software. From the NCBI database (www.ncbi.nlm.nih.gov), the mRNA sequences and partial coding sequences (CDS) of the selected genes were obtained and sent to Humanizing Genomics at Macrogen for the synthesis of designed primers.

Table6: Primers for stress-related genes and their specifications.

Target Genes	**Primer sequence 5' to 3'**		**TM °C**	**GC %**	**Accession NO**
HSP 70	F	GGACATCAGTCAGAACAAGAGG	62	50	KM369886.1
	R	TCTGGTGATGGACGAGTAGAA	62	47.6	
HSP 90	F	GGCAAGGACCTGAAGATC	57	55.56	KM091924.1

	R	CAGACTTGGCGATGGTAC	57	55.56	
β-Actin	F	ATGAAGATCCTGACCGAGAGA	62	47.6	EU 184877.1
	R	CTCGAAGTCAAGAGCCACATAG	62	50	

Moreover, isolation and quantification of RNAand synthesis of cDNA were conducted by adopted procedures mentioned in detail in the materials and methods section of experiment 2.

Real time – qPCR for HSP Gene expression

Initially, PCR conditions and cycle number were optimized for HSP genes. Each PCR reaction was run in standard of 20 µL reaction mixture 1.6 µl of diluted cDNA, 0.4 µl of forward and reverse primer, 10 µl of SYBRgreen and 7.6 µl of H_2O. RT - qPCR reaction condition included initial denaturation at 95°C for 10 minutes followed by 40 cycles at 95°C for 15 seconds and subsequently, 62°C for 15 secondsThe efficiency of PCR reaction for each gene was checked by plotting, standard curves with serial dilutions (1/10, 1/100 and 1/1000) of cDNA of a control sample. The mRNA levels of HSP70 and HSP90 were normalized to the expression of *L. rohita*beta actin(Housekeeping gene. The relative variations in gene expression were calculated by the standard ΔΔCTmethod (Pfaffl 2001).

Experiment 4. Comparative evaluation of disease resistance of different strains of *Labeorohita*

Study design

The procedure reported previously (Kamran et al., 2023) was adopted.Briefly,an experiment was conducted in triplicate and in separate tanks for each stock and fingerlings (average body weight, 20 to 22 g) of each stock were stocked in 9 fiberglass tanks (stocking density, of 2 g/L (20 fingerlings/tank). The first three tanks of each stock were marked as a control without exposure to pathogen, the other three for the evaluation of survival probability during 15 days of exposure, and the last three for the innate immunity indices and temporal expression of immune-related genes. Each fiberglass tank was well equipped with a proper aeration system for maintaining the DO level (6.0 ± 0.5 mg/L) and a water heater to maintain the temperature (27.0 ± 0.5 °C).

Challenge Test

After two weeks of acclimatization, all fiberglass tanks, except two of each stock, were exposed to a pathogenic strain of *Aeromonas hydrophila* (ATCC49140), freshly prepared culture from an infected L. *rohita* obtained from the Department of Microbiology, QAU. The fish were injected intraperitoneally 100 µL of the bacterial suspension at a concentration of 1.7 × 104 CFU/g fish, as reported by Sahu et al. (2011). The fish were kept for 15 days. During that period, mortality and disease symptoms were observed closely in each group. The survival probability of different stocks was calculated using Kaplan–Meier curves, while the log-rank test (Mantel-Cox) was used to determine the difference in survival time among the groups (Kamran et al., 2023). For the evaluation of health status and immunity after exposure to a pathogen, the fish left after the survival probability test were used. Fish remained in tanks for 56 hours, subsequently, anaesthetized with buffered MS222 and their blood was drawn and saved for hematological indices and separation of serum for immunity indices. Moreover, fresh blood was used for respiratory burst activity and phagocytic activity. For gene expression study, 9 fingerlings from the last two tanks of each stock (3 fingerlings /tank) were captured after 0, 56, 112, and 168-hours post-infection (PI), anesthetized, and dissected, and their liver samples (Kamran et al., 2023)were collected and kept separately in autoclaved Eppendorf tubes containing RNA Later™ and stored at −80˚C till the isolation of RNA.

Table7: Primers for immunity genes and their specifications

Target Genes	Primer Sequence 5' to 3'	TM ℃	CG%	Amplicon Size (bp)	E	Accession No.
complement component (C9)	F<TCGGCTTTACAGGCCAAGTC	58	55	110	98.1	KJ486471.1
	R<GTTCCTGTCATCTGTGGGGG	58	60			
Hepcidin	F<CTGCGGGCATTTTACTGAG	62	52.6	93	97.1	MF805810.1
	R <CAGCAAGGGGTTTTGAAGAC	62	50			
Transferrin	F<GGATGGCAGCAGGAAAAG	61	55.6	160	98.3	AM709723.1
	R <CCCAAAAGCAGCAGAAGAG	61	52.6			

bp, base pair; GC, guanine, and cytosine content; E, efficiency in percentage

Gene expression analysis post exposure

For the post exposure gene expression investigation, a tissue specimen was homogenized in chilled TRIzol® reagent with the assistance of a mortar and pestle, and total RNA extraction followed the TRIzol protocol (TRIzol®; by Life Technologies). The extracted RNA was dissolved in 30 µL of RNase-free water, with RNA quantity and quality assessed using NanoDrop® (BERTHOLD, Bad Wildbad, Germany). Subsequently, the cDNA synthesis procedure outlined by Murtaza et al. (2020) was adopted. RT-qPCR was then conducted utilizing a quantitative thermal cycler (MyGo Pro version 3.4.8). β-actin served for sample normalization and as a positive control. For gene quantification, specific primer pairs for each gene (Table 1.7) were designed via Oligo Explorer 1.1.2 software utilizing mRNA sequences sourced from NCBI (http://www.ncbi.nlm.nih.gov and synthesized by Humanizing Genomics Macrogen (Ahmad et al., 2020). PCR reactions were prepared in a total volume of 10 µL containing 0.5 µL of cDNA, 0.25 µL of each primer, 5 µL of SYBR Green (real-time PCR MMX (2×)), and 4 µL of injection water. Negative controls received water instead of cDNA. The RT-qPCR cycling conditions was as initial denaturation for 300 s at 94 ◦C, followed by 40 cycles of amplification and quantification (10 s at 94 ◦C, 30 s at the primer-specific annealing temperature, as indicated in Table 1.7), a pre-melting hold for 10 s at 94 ◦C, and high-resolution melting at 90 ◦C for 60 s and 15 s, respectively, with a heating rate of 0.05 per second. Primer efficiency was evaluated by serially diluting pooled cDNA (1/10, 1/100, 1/1000), with efficiency calculated utilizing the equation previously employed by Radonic et al. (2004) (Table 1.7). PCR runs were conducted in duplicate, with each sample analyzed in triplicate within each PCR. Relative expression levels were determined using the relative quantification method (Pfaffl, 2001).

Statistical analysis

The research region was mapped with ArcGIS 10.8. To assess variation in microsatellite loci, GENEPOP version 4.0 (Rousset, 2008) was used to quantify parameters such as the number of alleles per locus (NA), allelic frequency, observed heterozygosity (Ho), anticipated heterozygosity (He), and divergence from Hardy-Weinberg equilibrium (HWE). FSTAT version 2.9.3.2 (Goudet, 1995) was used to assess allelic richness (Ar) and the relevance of inbreeding coefficients within and within populations,

while Genetixver 4.05 was used to calculate the inbreeding coefficient (FIS) as an indicator of inbreeding.

A UPGMA dendrogram, according to Nei (1972), was constructed utilizing the R programming language to assess the relatedness among strains and the genetic variance by clustering. This analysis included the estimation of the number of alleles per locus, the allele size range at each locus, and inter-population disparities inferred from cluster formations using the inbreeding coefficient (FIS). Moreover, R-software facilitated the generation of a thermal polygon to delineate the intrinsic and acquired thermal zones characteristic of each strain. Additionally, a statistical analysis was conducted using a computerized statistical software package for social sciences (Version 20, Inc. Chicago, USA), employing one-way ANOVA followed by an LSD post hoc test to compare the expression of stress-related genes after exposure to mild and high temperature shocks. The statistical significance level for the entire study was set at 5%.

The obtained data was presented as mean ± SEM. Prior to conducting any statistical analysis, the homogeneity of variance and normality of data were assessed using Bartlett and Shapiro-Wilk's tests. One-way ANOVA was utilized to determine significant differences in growth performance, mortality percentage, thermal tolerance, survival, hematology, immunological indices, electrophoretic muscle protein profile, and gene expression level among different stocks of *L. rohita*. The LSD post hoc test was conducted at a 5 percent level of significance to determine the difference between the stocks. The computerized statistical software package for social sciences (SPSS, Software version 20, Inc. Chicago, USA) was used for statistical analysis. GraphPad Prism 5 was used to graphically represent the data.

RESULTS

Experiment 1. Assessment of genetic diversity among wild and captive bred *Labeorohita* through microsatellite markers and mitochondrial DNA

Genetic variability of microsatellite markers

Lr-28, Lr-29, and Lr-37 primers were effectively amplified by PCR, and all three microsatellite loci were identified as polymorphic, with a PIC value exceeding 0.5 (PIC > 0.5). The Lr-28 loci had a PIC value of 0.79, while the Lr-37 loci had a PIC value of 0.59. A total of 12 alleles were recognized at the three loci, with 5 alleles at the Lr-28 loci, 3 alleles at the Lr-29 loci, and 4 alleles at the Lr-37 loci. The number of alleles ranged from 3 to 5, with an average of 4.0 alleles / locus. Overall selected loci (Table 8), allelic size and frequency differed from sample to sample.

Table No 8. Genetic variations in different strains of *Labeorohita*at Lr-28, Lr-29, and Lr-37 locus

Strains	Parameters	Lr-28	Lr-29	Lr-37	Overall Mean
	PIC	0.79	0.63	0.59	**0.67**
	Alleles (bp)	175-168	170-166	161-155	**167.3**
	Ar	05.00	03.00	04.00	**4.00**
TMs (20)	H_o	0.891	0.791	0.691	**0.791**
	H_e	0.913	0.813	0.824	**0.85**
	F_{IS}	0.011	0.148	0.152	**0.103**
	HWE	0.004**	0.083	0.093	
WRs (20)	H_o	0.843	0.784	0.673	**0.766**
	H_e	0.892	0.797	0.799	**0.829**
	F_{IS}	0.017	0.167	0.310	**0.144**
	HWE	0.084	0.064	0.088	
MKs (20)	H_o	0.769	0.719	0.671	**0.723**

	H_e	0.872	0.839	0.844	**0.828**
	F_{IS}	0.106	0.237	0.325	**0.152**
	HWE	0.071	0.005**	0.076	
CMs (20)	H_o	0.791	0.721	0.652	**0.721**
	H_e	0.831	0.811	0.822	**0.821**
	F_{IS}	0.162	0.197	0.372	**0.223**
	HWE	0.081	0.051*	0.003**	
TKs (20)	H_o	0.736	0.636	0.643	**0.671**
	H_e	0.829	0.750	0.695	**0.758**
	F_{IS}	0.347	0.363	0.266	**0.245**
	HWE	0.052*	0.002**	0.004**	
SPs (20)	H_o	0.753	0.673	0.661	**0.695**
	H_e	0.862	0.810	0.773	**0.815**
	F_{IS}	0.201	0.289	0.292	**0.201**
	HWE	0.076	0.043*	0.047*	
USs (20)	H_o	0.796	0.740	0.664	**0.733**
	H_e	0.881	0.857	0.781	**0.839**
	F_{IS}	0.157	0.183	0.331	**0.223**
	HWE	0.098	0.001**	0.049*	
WKs (20)	H_o	0.854	0.743	0.670	**0.755**
	H_e	0.897	0.892	0.793	**0.860**
	F_{IS}	0.013	0.186	0.297	**0.148**
	HWE	0.083	0.075	0.043*	

Ar: Allelic richness, PIC: Polymorphic information content, H_o: Observed Heterozygosity, H_e: Expected Heterozygosity, F_{IS}: Fixation Index (Inbreeding coefficient), and conformity to Hardy-Weinberg equilibrium (HWE)

* Significant ($p < .05$) and ** highly significant ($p < .01$) departure from Hardy–Weinberg equilibrium.

Genetic diversity between strains and Hardy-Weinberg Equilibrium

The genetic variation observed among different strains is presented in Table 2. The Ho ranged from 0.671 to 0.791 for all strains, with TMs having the highest Ho and TKs having the lowest. Likewise, the He ranged 0.758 to 0.860, with the highest He of WKs, followed by TMs and TKs having the lowest He. TMs showed the highest genetic variability, while TKs showed the lowest compared to other strains, based on the overall mean value of all loci. The FIS value ranged from 0.103 to 0.245, with the lowest average FIS value for TMs followed by WRs and WKs, while TKs showed the highest average FIS value. Moreover, 12 of the 24 tests showed significant deviations from HWE. Genotype frequencies at all twelve loci across 24 tests showed an overall deviation from HWE (Table 2). CMs, TKs, SPs, and USs showed deviation at two loci each, TMs, MKs, and WKs at one locus, while WRs did not show deviation from HWE at any loci.

Genetic differentiation and inter-strain genetic structure

The study conducted pairwise comparisons between each strain (Table 9) and found significant differences in their genetic differentiation. For microsatellite markers, the FST value indicated that all strains showed differentiation. The FST value for all strains had an average range of 0.001 to 0.059, indicating a moderate to low level of population structure. The study revealed that the gene flow was highest between WKs and WRs and lowest between CMs and MKs. The highest FST value between CMs and MKs indicated that both strains do not share the allele and are remarkably different (FST= 0.059) while the lowest FST value demonstrated maximum gene flow between both strains (FST =0.001). Similarly, the genetic distance analysis based on the CO1 gene the highest gene flow (FST = 0.001) while the minimum gene flow between WRs and USs (0.068).

Table No 9: Pairwise F_{ST} (below diagonal) and P-values (above diagonal) among different strains of *Labeorohita*populations across all loci

	Microsatellite								mtDNA							
	CMs	**TKs**	**MKs**	**WRs**	**TMs**	**SPs**	**USs**	**WKs**	**CMs**	**TKs**	**MKs**	**WRs**	**TMs**	**SPs**	**USs**	**WKs**
CMs	-	0.001	0.017	0.002	0.001	0.016	0.002	0.001	-	0.017	0.017	0.001	0.013	0.022	0.011	0.003
TKs	0.011	-	0.0021	0.013	0.001	0.019	0.001	0.011	0.007	-	0.001	0.020	0.001	0.023	0.002	0.012
MKs	0.059	0.057	-	0.003	0.001	0.017	0.001	0.002	0.067	0.061	-	0.001	0.002	0.013	0.011	0.001
WRs	0.037	0.033	0.052	-	0.006	0.014	0.007	0.012	0.038	0.032	0.064	-	0.001	0.019	0.004	0.021
TMs	0.048	0.049	0.004	0.042	-	0.013	0.003	0.015	0.063	0.061	0.005	0.051	-	0.017	0.006	0.018
SPs	0.010	0.017	0.056	0.032	0.056	-	0.001	0.001	0.009	0.008	0.061	0.036	0.061	-	0.003	0.002
USs	0.053	0.045	0.020	0.049	0.021	0.045	-	0.013	0.067	0.063	0.050	0.068	0.040	0.061	-	0.016
WKs	0.020	0.020	0.052	0.002	0.048	0.022	0.019	-	0.035	0.031	0.059	0.004	0.057	0.034	0.055	-

Significant values at $p < .01$ (for microsatellite loci and mtDNA).

AMOVA for Microsatellites and Mitochondrial Marker (CO1) gene

The molecular variance analysis presented in (Table 10) revealed a minor genetic differentiation among strains using microsatellites (FST = 0.03524, p = 0.000) and COI gene (FST = 0.04464, p = 0.000). FIT of 0.471 for microsatellite markers and 0.343 for COI was noted. However,

the observed genetic variation (FIT) in the populations (microsatellite marker, 68.98%, and COI gene, 59.36%) is at the individual level within the strains rather than between them, and the differentiation is due to individuals within a population.

Table 10: Analysis of molecular variance (AMOVA) within and among eight strains.

Variation source	Variance component	Variation (%)	Fixation Parameters	Significance (P value)
Microsatellite Markers				
Among populations	0.030	4.51	F_{ST} = 0.03524	0.000
Among individuals within populations	0.621	26.56	F_{IS} = 0.17987	0.000
Within individuals	1.115	68.98	F_{IT} = 0.47154	0.000
mtDNA				
Among populations	0.038	7.63	F_{ST} = 0.04464	0.000
Among individuals within populations	0.700	33.01	F_{IS} = 0.18811	0.000
Within individuals	1.323	59.36	F_{IT} = 0.34312	0.000

Fisher's exact test

Fisher's exact test revealed significant variation among strains, as demonstrated in the results given in Table 11. The P-values for Microsatellite loci comparisons between different population pairs are detailed and an overall mean P-value is calculated by averaging across the various loci comparisons within each population pair. Noteworthy distinctions in allele frequencies are apparent in several population pairs, including CMs and TKs, TKs and WRs, MKs and TMs, WRs and TMs, WKs and SPs, and SPs and WRs ($P < 0.05$).

Table No 11. Fisher's Exact Test for Microsatellites allele homogeneity of all populations of *Labeorohita*

Population Pairs	**P-value at different Microsatellite Loci**			**Overall Mean**
	Lr-28	**Lr-29**	**Lr-37**	
CMs and TKs	0.0310	0.0787	0.0213	0.043
CMs and MKs	0.6582	0.000	0.000	0.219
CMs and WRs	0.4377	0.5214	0.320	0.426
CMs and TMs	0.300	0.410	0.222	0.310
TKs and MKs	0.2967	0.9343	0.127	0.452
TKs and WRs	0.0100	0.5674	0.0967	0.224
TKs and TMs	0.2241	0.1932	0.289	0.235
MKs and TMs	0.0783	0.0031	0.000	0.027
MKs and WRs	0.1231	0.1380	0.0162	0.092
WRs and TMs	0.0154	0.0123	0.0161	0.014
WKs and TMs	0.0185	0.0326	0.0000	0.017
WKs and SPs	0.0738	0.0000	0.0053	0.026
SPs and USs	0.5040	0.4232	0.4054	0.444
SPs and TMs	0.4210	0.1426	0.1729	0.245
SPs and WRs	0.0000	0.3254	0.0000	0.108

Relatedness through UPGMA dendrogram

The UPGMA dendrogram, illustrated in (Fig 5 A and B) and based on Nei‘s genetic distance (1972), demonstrated the genetic relatedness among all strains. The formation of two major clusters was depicted in Fig. 5A and B based on both microsatellite and mtDNA markers. One cluster comprised three strains, while the other consisted of five strains. Sub-clusters were further divided into each cluster. In the first sub-cluster, only the USs strain was present while TMs and MKs were in the other sub-cluster. The second sub-cluster was further divided into two clusters. One cluster had two strains, WRs, and WKs, while the other three strains, CMs, TKs, and SPs, were in the other.

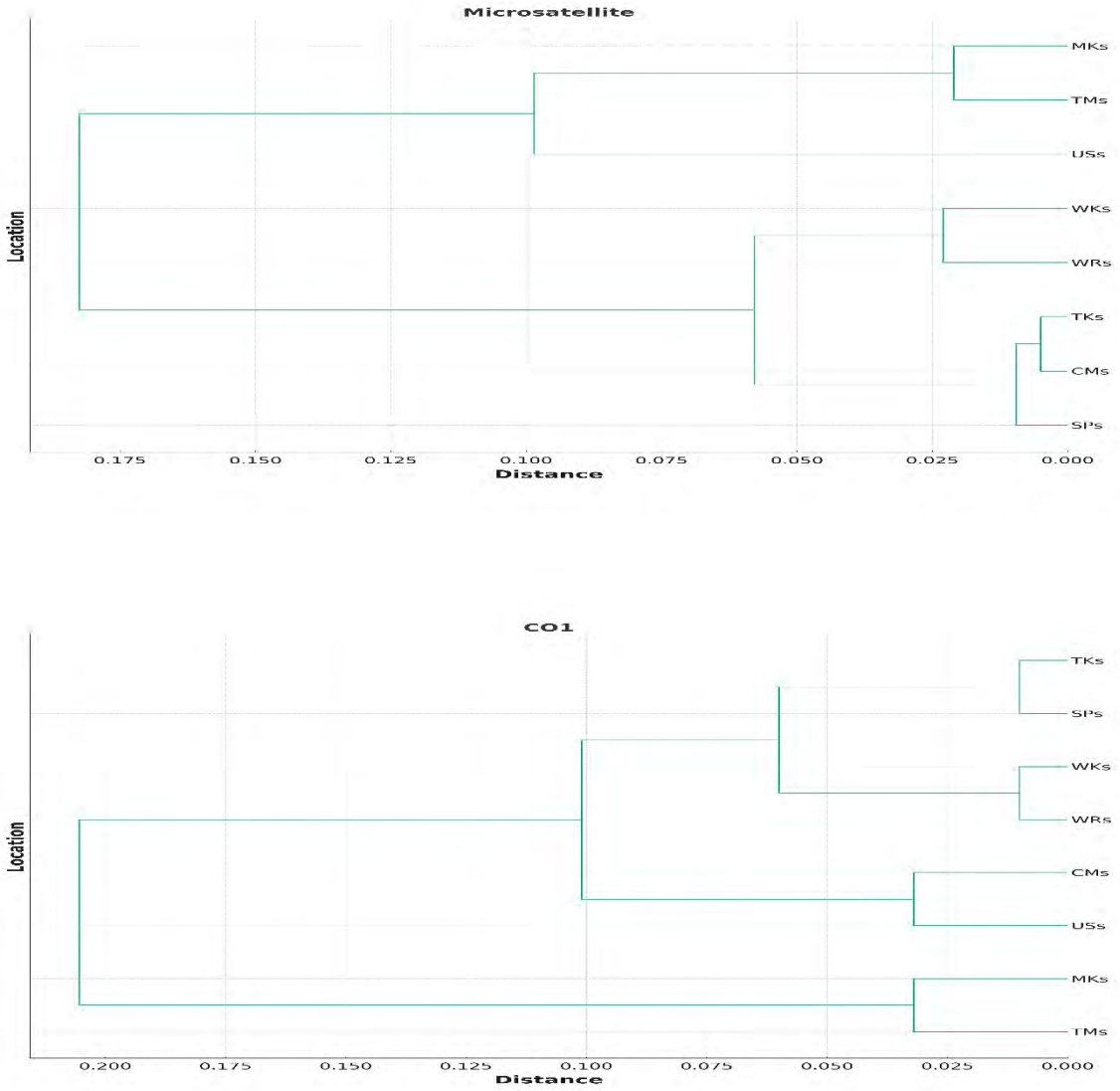


Fig. 5. Illustration of the genetic relationships among eight strains (TMs, WRs, MKs, CMs, TKs, SPs, USs, and WKs). (A) microsatellite markers based on genetic distances with clustering patterns reflecting relatedness. (B) mtDNA sequences of CO1 gene based on genetic distances with clustering patterns reflecting relatedness.

Experiment 2: Growth performance and innate immunity of advanced fry and fingerlingsof different strains of *Labeorohita*

Growth parameters of advanced fry in controlled conditions

The trial conducted for growth performance under semi-controlled conditions showed variation for different parameters (Table 12).

The One-way ANOVA showed significant variation (P<0.05) between strains of advanced fry for weight gain (n=3, ANOVA, $F_{7, 15}$ =31, p<0.05), weight gain percent (n=3, ANOVA, $F_{7, 15}$ =8.21, p<0.05), SGR (n=3, ANOVA, $F_{7, 15}$ =21, p<0.05), final biomass (n=3, ANOVA, $F_{7, 15}$ =58.3, p<0.05), FCR (n=3, ANOVA, $F_{7, 15}$ =194, p<0.05) and survival (n=3, ANOVA, $F_{7, 15}$ =31, p<0.05) between different strains. Upon close observation (LSD) of data ofgrowth performance of different strains, it was observed that TMs and MKs showed the highest weight gain in controlled conditions followed by WRs and WKs while USs showed followed closely. However, CMs and IBDs showed lower results with CMs being the lowest of all as shown in Table. 5.Similar results were also obtained for weight gain (%) and SGR where a similar trend was noted. However, FCR value was highest for CMs and IBDs while lowest FCR was noted for TMs. Moreover,the highest survival was noted in WKs while lowest survival was noted in CMs.

Table 12: Growth performance of all strains (advanced fry) in terms of weight gain, weight gain (%), SGR under semi-controlled conditions

Parameters	**Strains**							
	TMs	**WRs**	**MKs**	**CMs**	**IBDs**	**USs**	**SPs**	**WKs**
IBW (g)	1.49±0.03^{a}	1.49±0.06^{a}	1.49±0.02^{a}	1.49±0.04^{a}	1.49±0.06^{a}	1.49±0.07^{a}	1.49±0.09^{a}	1.49±0.08^{a}
FBW (g)	7.59±0.12^{a}	6.99±0.05^{b}	7.51±0.12^{a}	5.48±0.22^{e}	5.89±0.19^{de}	6.51±0.15^{c}	5.92±0.16^{d}	6.92±0.17^{b}
Weight gain(g)	6.10±0.15^{a}	5.53±0.09^{c}	6.04±0.14^{b}	3.00±0.18^{e}	4.40±0.16^{d}	5.05±0.17^{cd}	4.47±0.13^{d}	5.46±0.12^{c}
Weight gain (%)	409.4±8.87^{a}	378.1±8.80^{b}	410.20±7.22^{a}	202.70±5.7^{d}	295.30±6.63^{d}	346.6±7.32^{c}	308.30±6.84^{c}	374.00±8.31^{b}
SGR	3.54±1.04^{a}	3.25±1.12^{ab}	3.52±1.02^{a}	2.07±2.11^{c}	2.91±1.01^{bc}	3.09±1.93^{b}	3.00±1.31^{b}	3.24±1.43^{ab}
Initial biomass (g)	89.40±0.88^{a}	87.60±0.72^{b}	88.2±0.67^{ab}	88.80±0.76^{a}	89.40±0.68^{a}	87.60±0.78^{b}	87.00±0.83^{b}	87.60±0.69^{b}
Final biomass (g)	455.4±0.40^{a}	419.40±0.37^{ab}	450.60±0.23^{a}	329.40±0.55^{c}	353.40±0.10^{bc}	390.60±0.52^{b}	355.2±0.50^{bc}	415.20±0.41^{ab}
FCR	1.44±0.09^{e}	1.71±0.07^{d}	1.98±0.17^{c}	2.69±0.16^{a}	2.58±0.07^{a}	2.21±0.04^{b}	2.31±0.03^{b}	1.76±0.03^{d}
Survival (%)	91.67±0.42^{b}	91.67±0.95^{b}	88.33±0.71^{bc}	86.67±2.04^{c}	90.00±1.65^{b}	91.67±0.93^{b}	90.00±0.83^{b}	93.33±0.91^{a}

Data is presented as Mean± SE. Post hoc LSD test with ANOVA indicates a comparison among eight different strains. Superscripts letters abc shows a comparison between strains. Means sharing different superscripts are significantly different from each other (P < 0.05).

Haematological indices in controlled environment

One way ANOVA revealed significant differences in erythrocytes (n=9, ANOVA, $F_{7, 64}$=18.7, p<0.001, Table 13), leukocytes (n=9, ANOVA, $F_{7, 64}$=161,p<0.001, Table 6), haemoglobin (n=9, ANOVA, $F_{7, 64}$=9.10, p<0.001, Table 6), haematocrit (n=9, ANOVA, $F_{7, 64}$=26.9, p<0.001, Table 6), of all strains. The Post hoc test showed statistical comparable and significantly higher values of WBCs, Hb and HCT in WRs and TMs as compared to other strains. Furthermore, SPs and IBDs showed statistically comparable but lower level of WBCs, RBCs and HCT, however lowest level of HGB was observed in CMs.

Table 13: Haematological parameters of all strains after 30 days rearing under controlled environment

Strains	Parameters			
	WBC (10^3 µL^{-1})	**RBC (10^6µL)**	**HCT%**	**HGB (g dL^{-1})**
TMs	145.78 ± 5.21^{a}	2.45 ± 0.15^{a}	36.92 ± 2.98^{a}	11.84 ± 0.18^{a}
WRs	143.09 ± 4.73^{b}	2.55 ± 0.22^{a}	34.90 ± 2.85^{a}	11.71 ± 0.14^{a}
MKs	134.72 ± 3.68^{c}	2.41 ± 0.18^{ab}	30.92 ± 2.53^{ab}	10.72 ± 0.15^{b}
CMs	92.76 ± 3.92^{d}	1.55 ± 0.14^{c}	20.72 ± 1.02^{c}	7.82 ± 0.12^{d}
IBDs	96.45 ± 4.64^{d}	1.73 ± 0.16^{c}	24.49 ± 1.21^{c}	8.71 ± 0.11^{c}
USs	128.47 ± 3.58^{cd}	2.29 ± 0.27^{b}	28.92 ± 3.08^{b}	11.42 ± 0.14^{a}
SPs	105.83 ± 5.21^{d}	1.68 ± 0.32^{c}	25.34 ± 2.73^{b}	8.71 ± 0.11^{c}
WKs	141.97 ± 3.12^{b}	2.58 ± 0.18^{a}	33.05 ± 2.68^{a}	11.42 ± 0.14^{a}

Data is presented as Mean± SE. ANOVA followed by LSD post hoc test shows comparison between all strains. Superscripts letters abc shows comparison between strains. Means sharing different superscripts are significantly different from each other (P < 0.05).

Immunological indices in controlled conditions

The immunological indices of all stains reared under control conditions are presented in Table 14. All strains were significantly different in term of immunological indices:- Lysozyme (n=9, ANOVA, $F_{7, 64}$ =21, $p<0.05$), Total Protein (n=9, ANOVA, $F_{7, 64}$=28.7, $p<0.05$), IgM (n=9, ANOVA, $F_{7, 64}$ =17.1, $p<0.05$), Respiratory burst activity (n=9, ANOVA, $F_{7, 64}$ =17.8, $p<0.05$), Phagocytic activity (n=9, ANOVA, $F_{7, 64}$ =42.9, $p<0.05$), Phagocytic Index (n=9, ANOVA, $F_{7, 64}$=3.62 , $p<0.05$) and AST (n=9, ANOVA, $F_{7, 64}$ =33 $p<0.05$). Moreover, LSD test revealed the highest values of total protein contents, IgM level, lysozyme, Respiratory burst, and Phagocytic activity of WRs followed by TMs, while CMs and IBDs showed lowest values. The AST value also showed significant variation between different strain (AST (n=9, ANOVA, $F_{7, 64}$=33, $p<0.05$). TMs showed significantly lower ($P < 0.05$) level of AST while somewhat higher level was observed in CMs.

Table 14: Immunological indices of all strains of *Labeorohita* after 30 days rearing in controlled environment

Parameters	Strains								F-value	P-value
	TMs	WRs	MKs	CMs	IBDs	USs	SPs	WKs		
Total protein (mg/mL)	18.5±0.45^{a}	15.7±0.35^{c}	13.2±0.30^{d}	9.8±0.25^{e}	11.0±0.22^{f}	16.5±0.32^{b}	12.5±0.28^{d}	17.3 ± 0.34^{b}	67.8	0.000
IgM (mg/mL)	9.50 ± 0.22^{a}	7.80±0.18^{c}	6.50±0.15^{d}	4.60±0.12^{e}	5.20±0.15^{f}	8.20±0.20^{b}	6.80±0.18^{d}	8.70±0.22^{b}	56.3	0.001
Lysozyme activity (µg/mL)	5.80±0.45^{a}	4.20±0.30^{c}	3.60±0.25^{d}	2.40±0.20^{e}	2.90±0.18^{f}	4.50±0.28^{b}	3.20±0.23^{d}	4.80±0.32^{b}	28.1	0.005
Respiratory burst activity (OD 540 nm)	1.54±0.09^{a}	1.07±0.10^{a}	0.99±0.08^{b}	0.57±0.09^{d}	0.88±0.08^{c}	0.96±0.11^{b}	0.90±0.11^{bc}	1.34±0.14^{a}	8.0	0.001
Phagocytic activity (%)	81.01±1.32^{a}	71.2±1.01^{b}	69.5±1.20^{b}	55.7±1.31^{d}	56.1±1.10^{d}	60.1±1.87^{c}	68.32±1.12^{c}	78.7±1.20^{b}	57.4	0.000
Phagocytic index	1.91±0.09^{a}	1.82±0.05^{b}	1.59±0.04^{b}	1.34±0.08^{d}	1.40±0.05^{d}	1.56±0.07^{c}	1.61±0.17^{c}	1.70±0.09^{b}	29.2	0.001

Data presented as Mean ± SEM (n= 9). ANOVA followed by LSD post hoc test shows significant difference ($P<0.05$) among the strains. Similar lowercase letters in the rows shows no significant difference

Growth performance of fingerlings in communal ponds under semi-intensive culture system

In communal earthen ponds, fingerlings were reared for 3 months for assessment of the growth performance of fingerlings. Noted parameters showed significant difference in various strains, weight gain (n=3, ANOVA, $F_{7, 24}=13.7$, $p<0.05$), weight gain percent (n=3, ANOVA, $F_{7, 24}=2.64$, $p>0.05$), SGR (n=3, ANOVA, $F_{7, 24}=10$, $p<0.05$), final biomass (n=3, ANOVA, $F_{7, 24}=19.2$, $p<0.05$) and survival (n=3, ANOVA, $F_{7,}$

$_{24}$=7.6, p<0.05). Close observation using LSD showed variation in terms of weight gain as TMs showed highest weight gain followed by MKs, WRs, and WKs while lowest weight gain was observed for CMs, but it was statistically similar to IBDs, USs, and SPs. Similarly, weight gain (%) and SGR showed similar trends where TMs showed highest means followed by MKs, WRs, and WKs while lower results were obtained for CMs. However, survival (%) was statistically similar in CMs, IBDs, USs, and SPs while highest survival (%) was noted in TMs followed by MKs, WRs, and WKs. Growth data of all strains of fingerlings in communal ponds are shown in (Table 15).

Table 15: Growth performance of all strains (fingerlings) in terms of weight gain, weight gain (%), SGR in communal ponds under semi-intensive culture system

Parameters	**Strains**							
	TMs	**WRs**	**MKs**	**CMs**	**IBDs**	**USs**	**SPs**	**WKs**
IBW (g)	43.70±1.03^{a}	43.70±1.83^{a}	43.71±0.86^{a}	43.69±0.99^{a}	43.70±0.88^{a}	43.70±1.00^{a}	43.69±0.87^{a}	43.69±1.21^{a}
FBW (g)	261.67±7.42^{a}	228.48±8.50^{b}	231.67±6.01^{b}	184.39±9.48^{c}	195.44±1.22^{c}	186.31±7.21^{c}	184.11±9.11^{c}	227.91±6.63^{b}
Weight gain(g)	217.9±6.65^{a}	184.69±10.04^{b}	187.71±6.83^{b}	140.80±10.18^{c}	151.87±1.14^{c}	142.71±9.76^{c}	140.9±10.21^{c}	184.59±8.32^{b}
Weight gain (%)	497.6±10.71^{a}	421.6±42.54^{b}	427.50±25.68^{b}	323.40±33.24^{d}	348.50±11.64^{c}	327.1±21.18^{d}	326.2±16.76^{d}	425.8±18.90^{b}
SGR (%per day)	4.04±0.04^{a}	3.85±0.12^{b}	3.94±0.02^{b}	3.61±0.23^{d}	3.74±0.31^{c}	3.66±0.03^{d}	3.59±0.01^{d}	3.81±0.05^{b}
Initial biomass (g)	2626.2±25.84^{a}	2627.4±35.72^{a}	2646.2±86.04^{a}	2595.6±92.26^{a}	2594.2±45.68^{a}	2596.0±56.7^{a}	2566±60.23^{a}	2572.0±80.6^{a}
Final biomass (g)	15699.6±267^{a}	13664.8±183^{b}	13923.4±207^{b}	8678.1±284.93^{d}	10936.8±68.5^{c}	11132±200^{c}	8708.2±284^{d}	15877±198^{a}
Survival (%)	95.00±1.33^{a}	93.33±1.33^{b}	93.33±1.33^{b}	81.7±2.31^{c}	90.00±1.33^{c}	91.70±1.23^{c}	90.00±2.01^{c}	93.33±2.32^{b}

Data is presented as Mean± SE. ANOVA followed by LSD post hoc test shows a comparison between five strains. Superscripts letters abc shows a comparison between strains. Means sharing different lowercase letters are significantly different from each other (P < 0.05).

Haematological indices in semi-controlled environment

One way ANOVA revealed significant differences in erythrocytes (n=9, ANOVA, $F_{7, 64}$=18.7, p<0.001, Table 16), leukocytes (n=9, ANOVA, $F_{7, 64}$=161,p<0.001, Table 9), haemoglobin (n=9, ANOVA, $F_{7, 64}$=9.10, p<0.001, Table 9), haematocrit (n=9, ANOVA, $F_{7, 64}$=26.9, p<0.001, Table 9), of all strains. The Post hoc test showed statistical comparable and significantly higher values of WBCs, Hb and HCT in WRs and TMs as compared to other strains. Furthermore, CMs and IBDs showed statistically comparable but lower level of WBCs, RBCs and HCT, however lowest level of HGB was observed in MKs.

Table 16: Haematological parameters of all strains after 90 days rearing in communal ponds under semi-intensive culture system

Strains	Parameters			
	WBC (10^3 µL^{-1})	**RBC (10^6µL)**	**HCT%**	**HGB (g dL^{-1})**
TMs	151.95±7.63^{a}	2.51±0.12^{b}	38.17±4.04^{a}	12.06±0.22^{a}
WRs	149.32±6.02^{a}	2.67±0.19^{a}	36.06±3.75^{a}	11.93±0.12^{a}
MKs	138.53±4.89^{b}	2.53±0.27^{b}	31.28±3.08^{b}	10.83±0.13^{b}
CMs	99.85±4.59^{c}	1.64±0.20^{d}	21.28±1.21^{c}	8.12±0.10^{d}
IBDs	102.29±6.16^{c}	1.82±0.21^{d}	25.51±1.43^{c}	9.10±0.12^{c}
USs	134.56±4.14^{b}	2.38±0.32^{c}	29.32±4.13^{b}	9.87±0.11^{c}
SPs	110.32±6.43^{c}	1.76±0.41^{d}	26.45±3.12^{bc}	9.00±0.09^{c}
WKs	147.51±3.12^{a}	2.62±0.12^{a}	35.14±3.33^{a}	11.68±0.12^{ab}

Data is presented as Mean± SE. LSD post hoc test with ANOVA shows comparison between all strains. Superscripts letters abc shows comparison between strains. Means sharing different lowercase letters are significantly different from each other ($P < 0.05$).

Immunological indices after rearing in Communal ponds under semi-intensive culture system

Immune response parameters of all strain after 90 days rearing in communal ponds is shown in Table 17. Most of indices showed significant difference between strains like Total Protein (n=9, ANOVA, $F_{7,64}$= 3.96, $p<0.05$), Lysozyme (n=9, ANOVA, $F_{7,64}$= 27.3, $p<0.05$), IgM (n=9, ANOVA, $F_{7,64}$=23.4, $p<0.05$),Respiratory burst (n=9, ANOVA, $F_{7,64}$=13.6, $p<0.05$), Phagocytic activity (n=9, ANOVA, $F_{7,64}$=83.2, $p<0.05$), Phagocytic Index (n=9, ANOVA, $F_{7,64}$=17.6, $p<0.05$) and AST (n=9, ANOVA, $F_{7,64}$=13.2, $p<0.05$).The close observation of data indicated improved status of WRs followed by TMs, while IBDs and CMs showed weak immune response. For instance, peak levels of total protein, lysozyme activity, respiratory burst activity and phagocytic activity were observed in WRs strain followed by TMs, while both IBDs and CMs showed lowest levels of all studied parameters.

Table 17: Immunological indices of all strains of *Labeorohita* after three months rearing in communal pond under semi-intensive culture system

Parameters	Strains								F-value	P-value
	TMs	WRs	MKs	CMs	IBDs	USs	SPs	WKs		
Total protein (mg/mL)	22.3±0.72^{a}	17.4±0.35^{c}	17.1±0.35^{c}	15.7±0.28^{d}	14.8±0.33^{d}	18.8±0.13^{b}	18.1±0.18^{b}	17.1±0.61^{c}	65.2	0.005
IgM (mg/mL)	11.32±0.31^{a}	8.63±0.32^{d}	8.11±0.32^{d}	6.52±0.34^{e}	5.67±0.38^{e}	10.1±0.22^{b}	9.23±0.31^{c}	8.49±0.42^{d}	51.0	0.002
Lysozyme activity (µg/mL)	4.99±0.65^{b}	7.19±0.12^{a}	6.98±0.12^{a}	3.12±0.75^{c}	3.99±0.34^{bc}	2.32±0.42^{d}	2.25±0.37^{d}	7.06±0.17^{a}	21.4	0.000
Respiratory burst activity (OD 540 nm)	1.13±0.12^{a}	0.98±0.11^{a}	0.82±0.11^{b}	0.47±0.05^{d}	0.72±0.06^{c}	0.86±0.02^{b}	0.75±0.14^{bc}	0.90±0.12^{a}	6.57	0.000
Phagocytic activity (%)	75.93±1.98^{a}	67.1±1.31^{b}	66.2±1.31^{b}	48.1±1.12^{d}	47.7±0.79^{d}	54.2±1.56^{c}	57.2±1.09^{c}	66.6±1.01^{b}	51.1	0.001
Phagocytic index	1.87±0.05^{a}	1.61±0.08^{b}	1.52±0.08^{b}	1.21±0.04^{d}	1.33±0.07^{d}	1.43±0.05^{c}	1.41±0.17^{c}	1.59±0.10^{b}	21.0	0.000

Data presented as Mean ± SEM (n= 9). ANOVA followed by LSD post hoc test shows significant difference ($P<0.05$) among the strains. Similar lowercase letters in the rows shows no significant difference.

Antioxidant enzymatic activity

The results reveal distinctive patterns in enzymatic activities among the strains (Table 18). TMs exhibit the highest SOD (20.70±0.06 U/mg protein) and CAT (185.90±2.01 U/mg protein) values followed by WRs.

Meanwhile, MKs, CMs, IBDs, USs, SPs, and WKs display progressively lower SOD and CAT values. Similar trends are observed for POD and GST activities, with TMs consistently demonstrating the highest values, followed by WRs and WKs. Conversely, CMs consistently exhibit the lowest activities for all enzymes. Overall, the table underscores strain-specific variations in antioxidant enzyme activities.

Table 18: Comparison of SOD, CAT, POD, and GST activity (U/mg protein) in gills among eight strains of *Labeorohita*

Strain	SOD	CAT	POD	GST
TMs	20.70±0.06[a]	185.90±2.01[a]	1.58±0.07[a]	120.72±1.58[a]
WRs	20.34±0.04[b]	183.75±2.45[b]	1.55±0.05[b]	118.90±1.70[b]
MKs	19.90±0.02[c]	181.50±3.11[c]	1.51±0.03[c]	116.10±1.90[c]
CMs	18.80±0.01[e]	177.20±3.15[e]	1.42±0.03[e]	112.50±2.30[e]
IBDs	18.95±0.01[e]	178.30±3.21[e]	1.43±0.03[e]	113.40±2.20[e]
USs	19.45±0.01[d]	180.50±3.34[d]	1.47±0.03[d]	115.20±2.00[d]
SPs	19.38±0.01[d]	179.40±3.28[d]	1.46±0.03[d]	114.30±2.10$_{d}$
WKs	20.29±0.03[b]	182.60±2.89[b]	1.55±0.04[b]	118.00±1.80[b]

Values are presented as mean ± standard deviation. Strains with the same superscript letter (a or b) within a parameter indicate statistically similar results.

Proximate composition

The proximate composition of all stains reared under control conditions are presented in Table. 19. All strains were significantly different in term of proximate composition:- Proteins (n=3, ANOVA, $F_{7,24}$ =25.1, p<0.001),Fats (n=3, ANOVA, $F_{7,24}$=19.9, p<0.001),Ash content (n=3, ANOVA, $F_{7,24}$ =21.5, p<0.001) and Carbohydrates (n=3, ANOVA, $F_{7,24}$ =16.6 p<0.001). Moreover, LSD test revealed the highest values of proteins and carbohydrates by TMs, while CMs and IBDs showed lowest values. Meanwhile, TMs showed lowest values of fats and ash content, but highest values were showed by CMs and IBDs.

Table 19: Proximate composition (Mean ± SD, n = 3) of different strains of *L rohita*after 90 days rearing in communal ponds under semi-intensive culture system

	Strains									
	TMs	**WRs**	**MKs**	**CMs**	**IBDs**	**USs**	**SPs**	**WKs**	**F-value**	**P-value**
Moisture (%)	70.32±2.00^{f}	71.31±3.31^{e}	71.45±2.15^{e}	75.45±3.2^{a}	74.89±2.1^{b}	73.32±1.4^{c}	73.78±1.2^{c}	72.12±1.7^{d}	10.6	.009
Crude Protein (%)	21.22±2.12^{a}	20.12±2.54^{b}	18.32±2.67^{cd}	16.66±1.8^{e}	16.78±2.8^{e}	17.14±1.9^{d}	17.89±2.2^{d}	19.32±2.7^{c}	11.1	.001
Fat (%)	1.65±1.4^{c}	1.69±1.1^{c}	1.89±1.0^{c}	3.32±1.8^{a}	3.11±1.6^{a}	2.12±1.3^{b}	2.16±1.9^{b}	1.75±1.6^{c}	1.63	.212
Ash (%)	3.76±0.98^{c}	3.91±1.12^{c}	4.00±0.97^{c}	6.11±1.31^{a}	6.06±1.11^{a}	5.32±0.97	5.45±1.14^{b}	3.23±1.11^{b}	6.61	.001

One-way ANOVA followed by LSD Post Hoc test shows a pairwise comparison between stocks. Alphabets on mean values are significantly showing difference (P < 0.05) between strains.

Gene expression

In analyzing the gene expressions of Myogenin, IGF-II, MyoD, and Preproghrelin across all strains (Fig. 6-15), distinct patterns emerged. Myogenin exhibited significantly higher expression in TMs compared to WRs, MKs, and WKs, as indicated by one-way ANOVA ($n = 9$, ANOVA, $F7, 64 = 230.3$, $p = 0.000$). Similarly, IGF-II expression (Fig. 7, 8) revealed TMs with the highest levels, followed by WRs and WKs, MKs, IBDs, USs, CMs, and SPs, with significant differences among strains ($n = 9$, ANOVA, $F7, 64 = 313.6$, $p = 0.000$). Moving to MyoD (Fig. 9), TMs exhibited the highest expression, followed by MKs, WRs, WKs, IBDs, USs, CMs, and SPs, with significant strain variations detected ($n = 9$, ANOVA, $F7, 64 = 145.6$, $p = 0.000$). In the case of Preproghrelin (Fig. 10, 11), TMs showcased the highest expression, followed by WRs and WKs, MKs, USs, IBDs, SPs, and CMs, with significant differences ($n = 9$, ANOVA, $F7, 64 = 246.1$, $p = 0.000$). Post hoc analysis indicated significantly higher IGF-II and Preproghrelin expressions in TMs and MKs compared to other strains after 90 days of communal pond rearing.

Similarly, one-way ANOVA was employed to observe gene expression in CT-Muscle, CT-Liver, Lysozyme-G, NKEF and TNF-alpha. Notably, CT-Muscle showed up-regulation in TMs compared to all other strains, with the lowest expression in CMs. CT-Liver exhibited a slightly different trend, with TMs and WRs showing peak expression, closely followed by MKs, while CMs displayed lower expression. Lysozyme-C and Lysozyme-G expressions were higher in TMs and MKs, followed by WRs and IBDs, while CMs showed lower expression. TNF-α expression was significantly higher in WRs, followed by TMs and MKs, with lower expression in CMs. Similarly, for NKEF, the expression was higher in TMs followed by WRs and WKs and MKs while rest of the strains showed intermediate results.

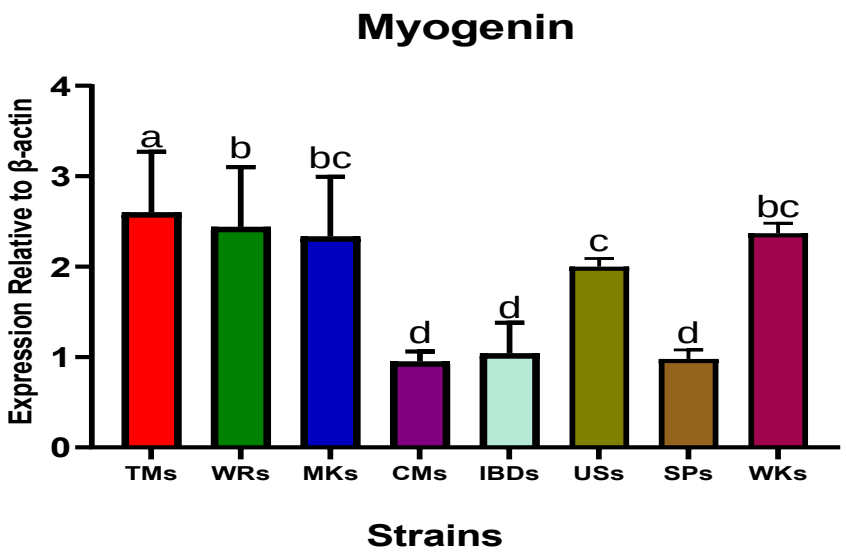


Figure 6. Expression of Myogenin gene relative to β-actin in the liver of different strains of *L. rohita*

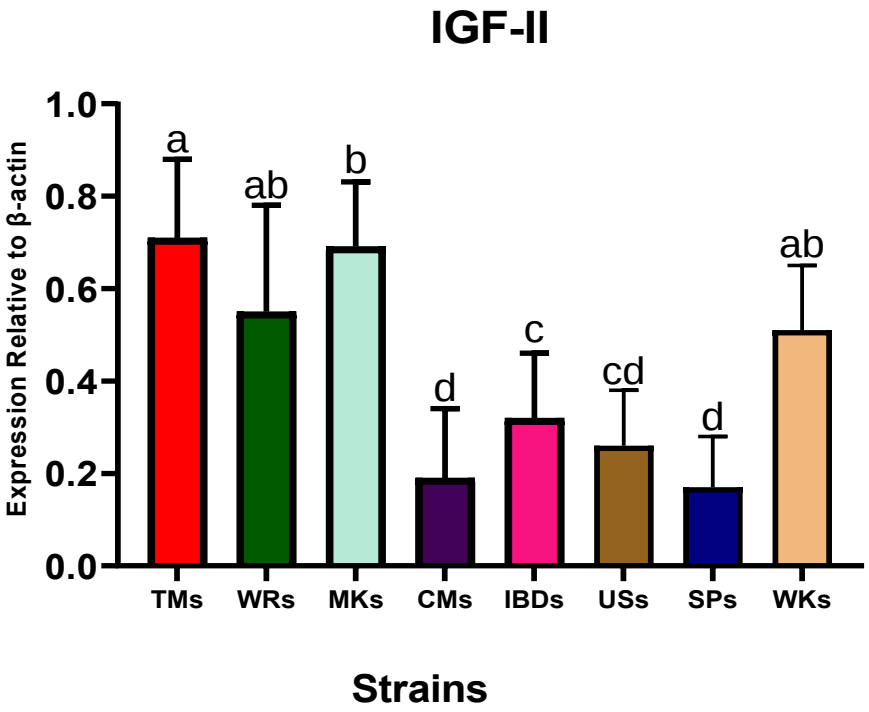


Figure 7. Expression of IGF-II gene in liver relative to β-actin in different strains of *L. rohita*

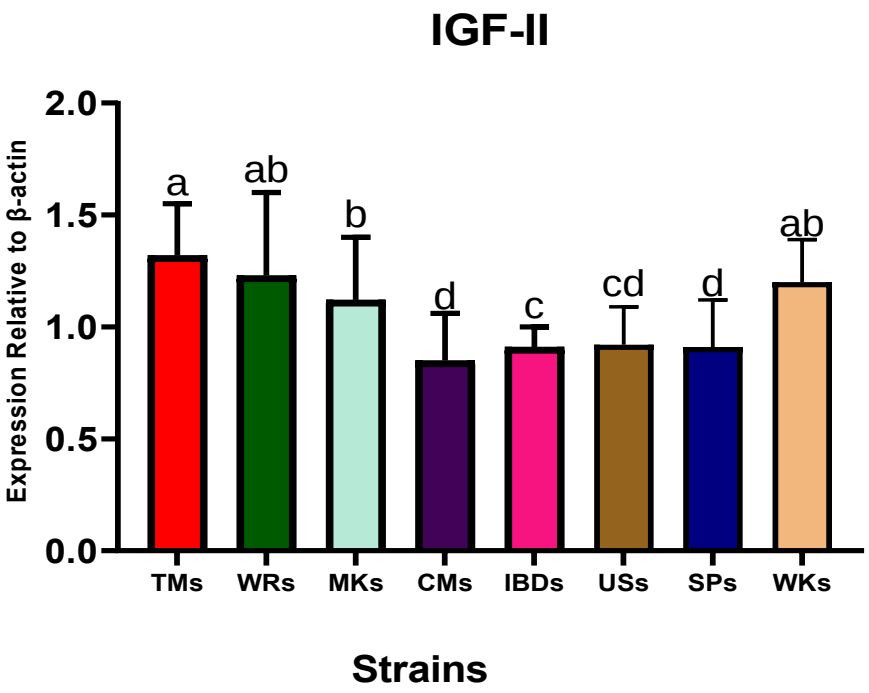


Figure 8. Expression of IGF-II gene in muscle relative to β-actin in different strains of *L. rohita*

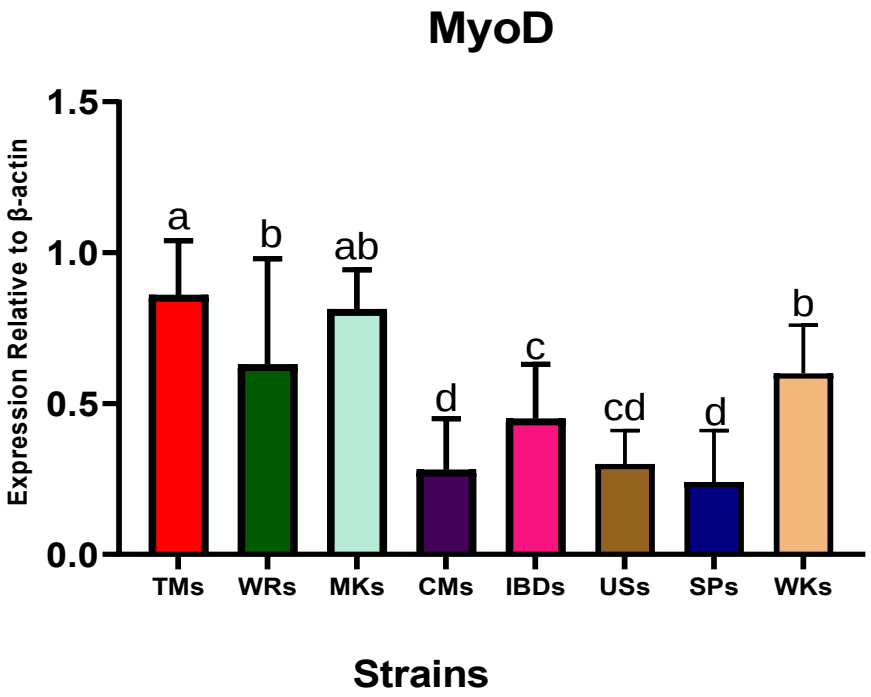


Figure 9. Expression of MyoD gene relative to β-actin in the liver of different strains of *L. rohita*

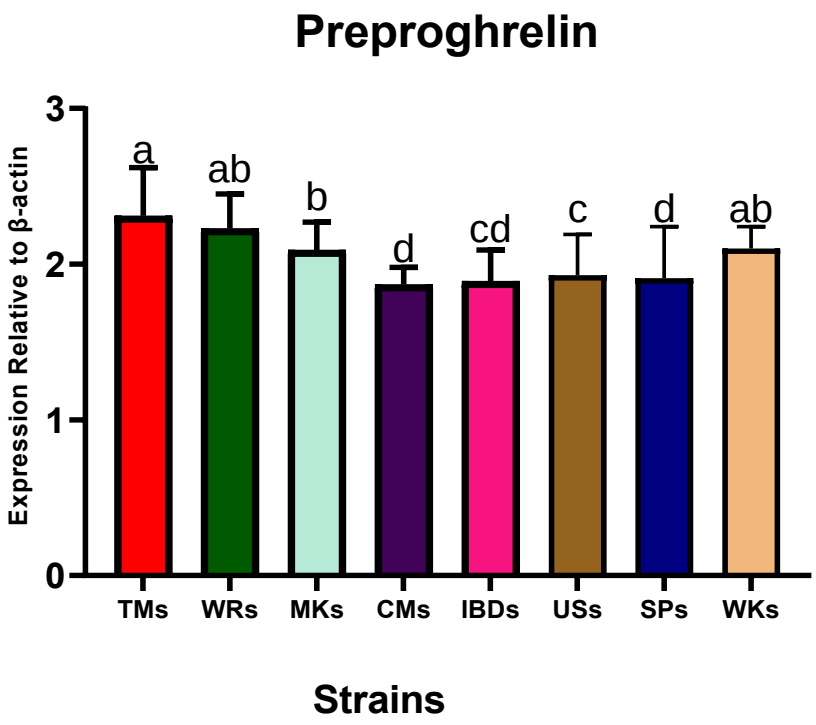


Figure10.Expression of Preproghrelin gene in liver relative to β-actin in different strains of *L. rohita*

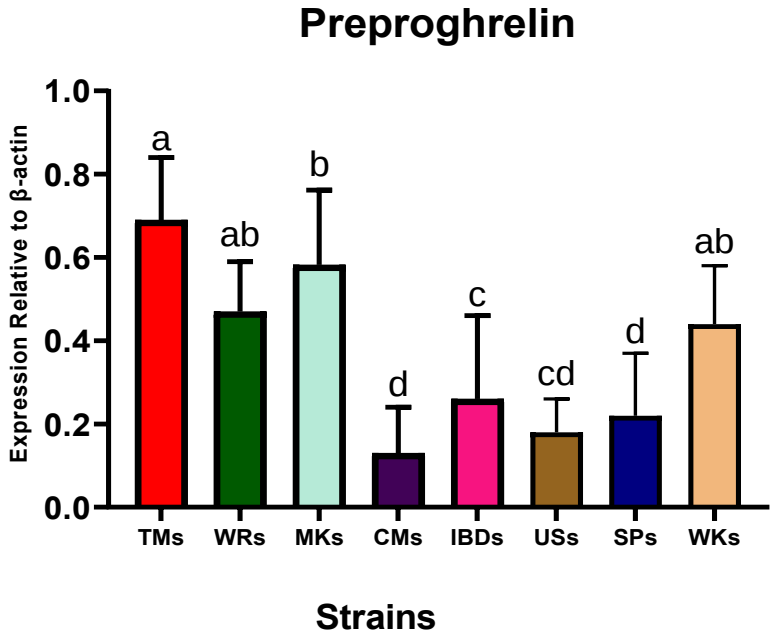


Figure 11. Expression of Preproghrelin gene in muscle tissues relative to β-actin in different strains of *L. rohita*

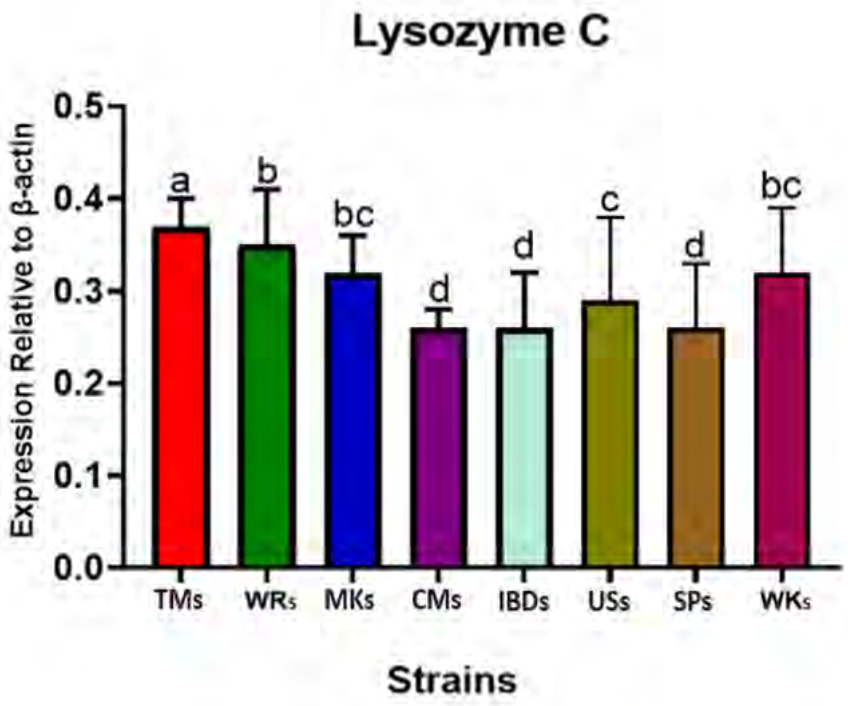


Figure 12. Expression of Lysozyme C gene relative to β-actin in liver of different strains of *L. rohita*

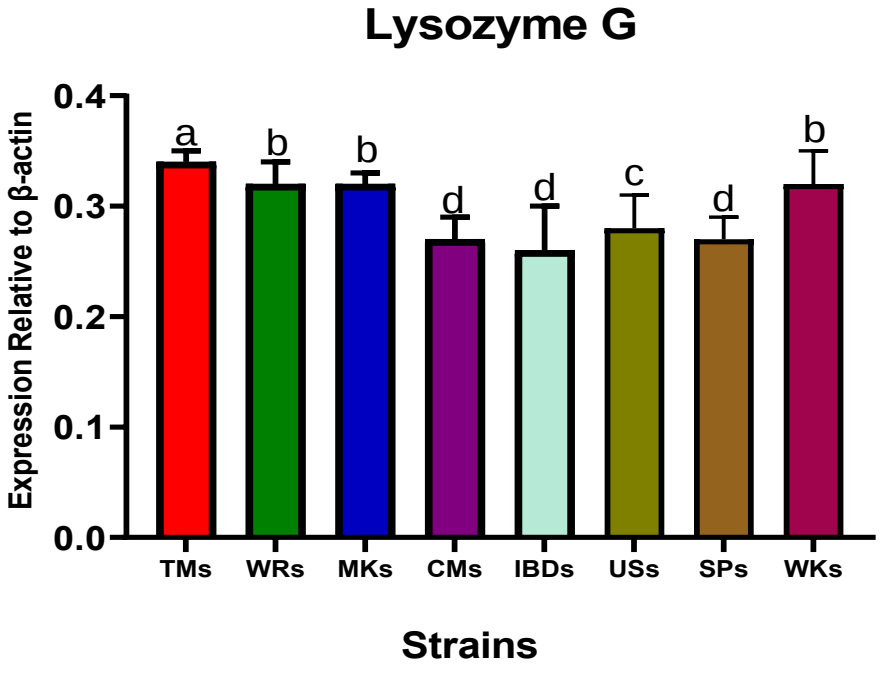


Figure 13. Expression of Lysozyme G gene relative to β-actin in liver of different strains of *L. rohita*

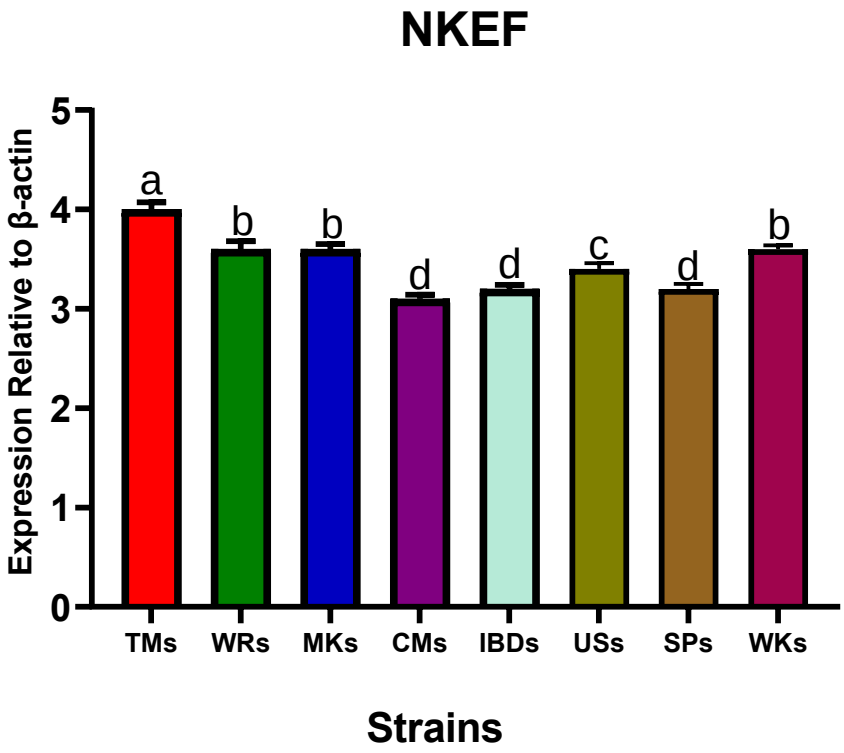


Figure 14. Expression of NKEF gene relative to β-actin in the liver ofdifferent strains of *L. rohita*

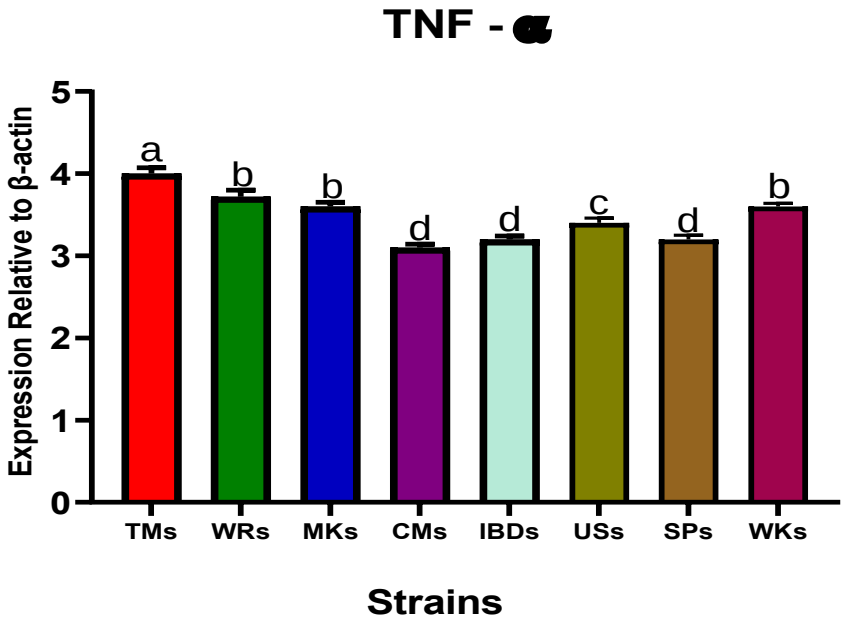


Figure 15. Expression of NKEF gene relative to β-actin in liver of different strains of *L. rohita*

Experiment 3: Comparative effect of acclimated temperature on thermal tolerance, and expression of HSP genes in different strains of *Labeorohita*

Thermal Tolerance

Significant variations ($P < 0.05$) in thermal tolerance were observed among the strains, as determined by a one-way ANOVA followed by an LSD post hoc test, for both CTMin and CTMax. The results are presented in Table 20. For CTMin (°C), among the eight strains evaluated, IBDs demonstrated the best performance, followed by WRs, SPs, WKs, MKs, USs, CMs, and TMs. The ranking was determined based on the lower values of CTMin (°C), indicating higher cold tolerance. The statistical analysis revealed a significant F ratio of 197 ($p < 0.001$), indicating a significant difference in the cold tolerance among the strains suggesting that IBDs exhibited the highest cold tolerance among the tested strains, while TMs showed the least cold tolerance.

Similarly, for CTMax (°C), among the eight strains evaluated, TMs demonstrated the best performance, followed by WRs, WKs, MKs, SPs, USs, CMs, and IBDs. The ranking was determined based on the higher values of CTMax (°C), indicating greater heat tolerance. The statistical analysis revealed a significant F ratio of 71.1 ($p < 0.001$), indicating a significant difference in heat tolerance among the strains. The low p-value ($p = 0.000$) further confirms the statistical significance of these findings.

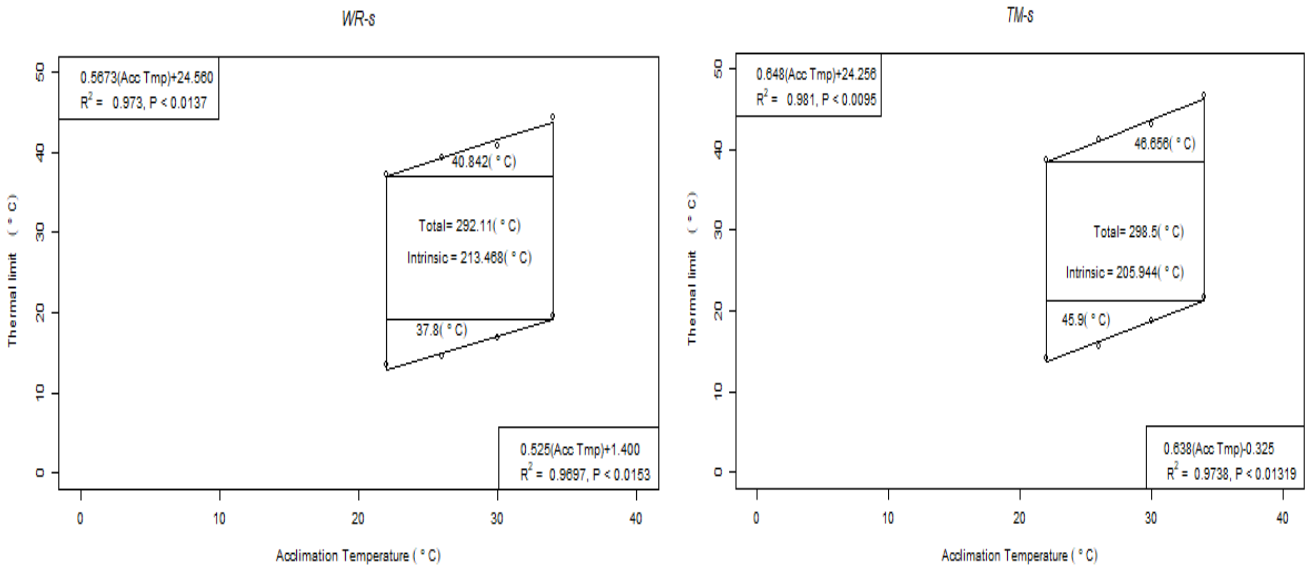
Table 20: Critical thermal Maxima CTMax and critical thermal Minima CTMin of all strains of *L. rohita* after acclimation to different temperature

Acclimation temperature (22°C)								
	Strains							
Parameters	**TMs**	**WRs**	**MKs**	**CMs**	**IBDs**	**USs**	**SPs**	**WKs**
CT_{Max} (°C)	38.67±0.39^{a}	37.27±0.38^{b}	37.40±0.23^{b}	36.53±0.35^{c}	35.63±0.27^{d}	36.32±0.25^{c}	36.45±0.32^{c}	37.01±0.37^{bc}
CT_{Min} (°C)	14.2±0.20^{a}	13.4±0.23^{b}	12.6±0.26^{c}	10.2±0.22^{d}	10.0±0.09^{d}	13.5±0.11^{b}	10.5±0.19^{d}	13.0±0.21^{bc}
Acclimation temperature (26°C)								
	Strains							
Parameters	**TMs**	**WRs**	**MKs**	**CMs**	**IBDs**	**USs**	**SPs**	**WKs**
CT_{Max} (°C)	41.20±0.15^{a}	39.3±0.18^{b}	40.3±0.07^{a}	38.73±0.29^{c}	37.4±0.11^{d}	40.3±0.21^{a}	37.98±0.17^{d}	39.5±0.17^{b}
CT_{Min} (°C)	15.5±0.17^{a}	14.5±0.20^{b}	14.9±0.14^{ab}	13.6±0.16^{c}	12.3±0.18^{d}	14.1±0.12^{b}	13.8±0.09^{c}	14.2±0.13^{b}
Acclimation temperature (30°C)								
	Strains							
Parameters	**TMs**	**WRs**	**MKs**	**CMs**	**IBDs**	**USs**	**SPs**	**WKs**
CT_{Max} (°C)	43.03±0.23^{a}	40.9±0.24^{a}	41.97±0.15^{a}	39.3±0.15^{b}	38.0±0.41^{c}	41.43±0.40^{ab}	39.7±0.35^{b}	40.2±0.29^{b}
CT_{Min} (°C)	18.8±0.09^{a}	16.9±0.12^{bc}	17.4±0.13^{b}	15.6±0.16^{cd}	14.5±0.15^{d}	17.8±0.11^{b}	15.0±0.10^{cd}	16.2±0.14^{c}
Acclimation temperature (34°C)								
	Strains							
Parameters	**TMs**	**WRs**	**MKs**	**CMs**	**IBDs**	**USs**	**SPs**	**WKs**
CT_{Max} (°C)	46.70±0.19^{a}	44.3±0.20^{b}	45.63±0.28^{a}	42.3±0.20^{c}	40.4±0.15^{c}	45.1±0.21^{ab}	42.7±0.20^{bc}	44.5±0.18^{b}
CT_{Min} (°C)	21.6±0.15^{a}	19.6±0.15^{b}	20.1±0.26^{b}	18.4±0.07^{c}	17.1±0.09^{d}	21.0±0.08^{a}	18.5±0.19^{c}	19.3±0.12^{b}

Thermal Polygons

For TMs, the polygon equation (0.692 * Acclimation Temperature + 24.256) revealed a wide thermal range, with an upper area of 46.656°C and a lower area of 45.9°C. WRs exhibited a narrower thermal range (polygon equation: 0.5673 * Acclimation Temperature + 24.560), with an upper area of 40.842°C and a lower area of 37.8°C. CMs displayed distinct thermal characteristics with a wide range (polygon equation: 0.447 * Acclimation Temperature + 26.699), having an upper area of 32.184°C and a lower area of 47.88°C.MKs demonstrated a balanced thermal range (polygon equation: 0.659 * Acclimation Temperature + 22.873) with an upper area of 47.448°C and a lower area of 45°C. IBDs had a narrower thermal range (polygon equation: 0.3727 * Acclimation Temperature + 27.4205) with an upper area of 26.838°C and a lower area of 42.3°C. USs exhibited a balanced range (polygon equation: 0.6868 * Acclimation Temperature + 21.558) with an upper area of 49.446°C and a lower area of 47.16°C. SPs, like USs, displayed a balanced thermal range (polygon equation: 0.5118 * Acclimation Temperature + 24.8785), having an upper area of 36.846°C and a lower area of 45.36°C.

The R2 values for all strains ranged from 0.922 to 0.981, indicating a strong correlation between acclimation temperature and thermal tolerance. The polygons are given below in Fig 12 (A-H).



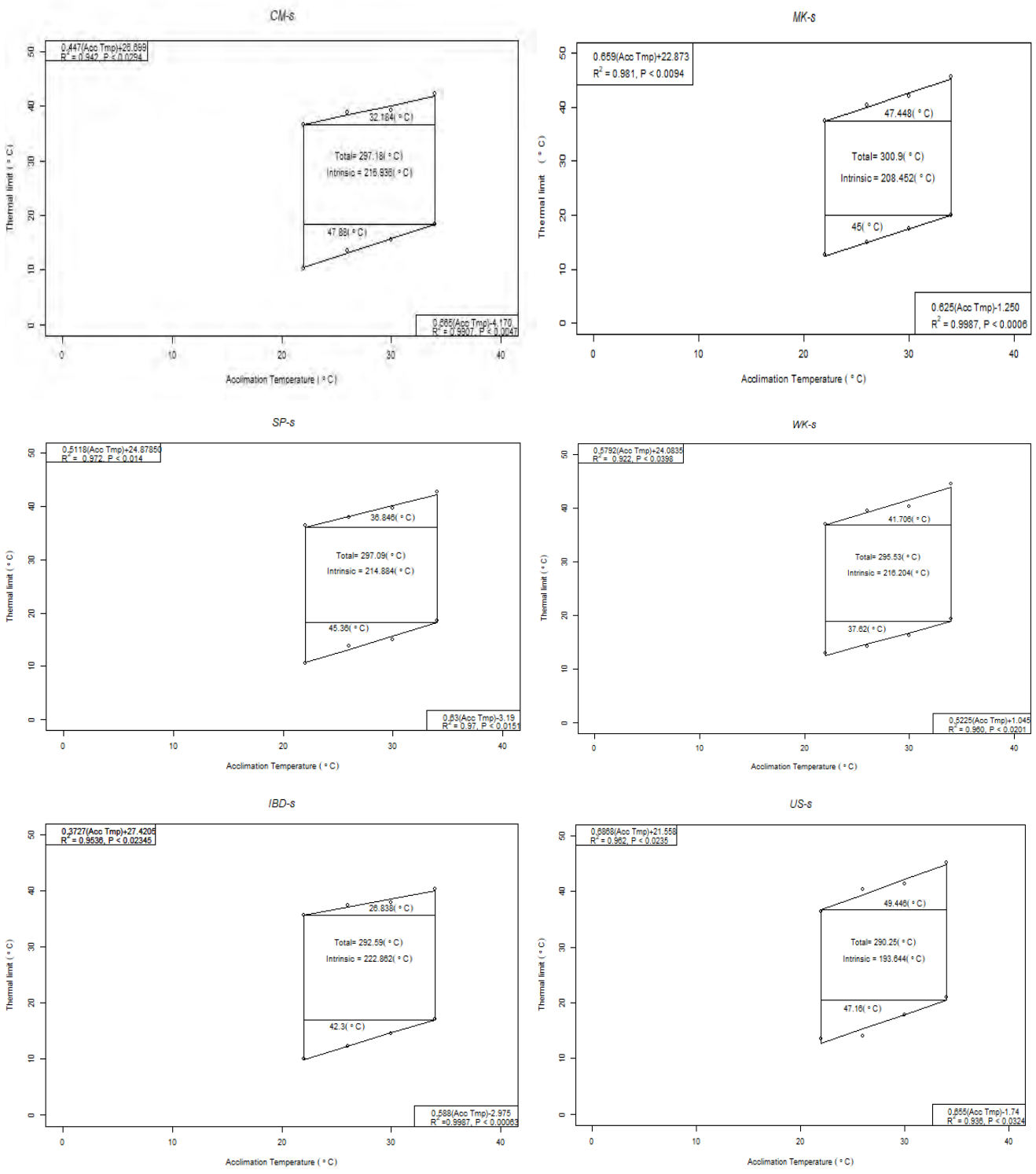


Fig 16: Thermal polygons showing the thermal range of each strain. WRs (A), TMs (B), CMs (C), MKs (D), SPs (E), WKs (F), IBDs (G), USs (H).

Expression of HSP90 and HSP70 genes

To investigate the impact of temperature stress on gene expression, two experimental groups were exposed to low (13°C) and high (37°C) temperatures, while a control group was maintained at 26°C (Table 21). Comparative analysis of gene expression levels revealed significantly increased expression of both HSP90 and HSP70 genes in the experimental groups compared to the control group. Importantly, the upregulation of the HSP70 gene was found to be significantly greater than that of the HSP90 gene. Statistical analysis ($P<0.05$) revealed significant differences among all groups for both HSP90 and HSP70 gene expression.

Table 21: Critical thermal minima (CTMin) and Critical thermal maxima (CTMax) of all strains of *Labeorohita* acclimated to 26°C after three months rearing in the communal pond under a semi-intensive culture system

Acclimation temperature (26°C)										
Parameters	**Strains**								**F ratio**	**P value**
	TMs	**WRs**	**MKs**	**CMs**	**IBDs**	**USs**	**SPs**	**WKs**		
CTMin (°C)	13.45±0.09^{a}	13.00±0.03^{b}	12.93±0.07^{b}	12.54±0.05^{c}	11.30±0.03^{d}	13.31±0.06^{a}	12.50±0.06^{c}	12.61±0.03^{c}	197	0.001
CTMax (°C)	41.3±0.06^{a}	40.23±0.04^{b}	39.11±0.07^{c}	38.5±0.02^{d}	36.5±0.03^{f}	40.3±0.04^{b}	38.76±0.04^{d}	39.79±0.05^{c}	71.1	0.000

Data presented as Mean ± SEM (n= 6). ANOVA followed by LSD post hoc test shows a significant difference ($P<0.05$) among the strains. Similar lowercase letter in the rows shows no significant difference.

Specifically, for HSP90 gene expression analysis, the groups were denoted as follows: control group (n=8, ANOVA, $F_{7, 56} = 161$, $P = 0.0000$), low thermal stress group (13°C) (n=8, ANOVA, $F_{7, 56} = 143$, $P = 0.0000$, Fig 13), and high thermal stress group (37°C) (n=8, ANOVA, $F_{7, 56} = 145$, $P = 0.0000$). Similarly, for HSP70 gene expression analysis, the groups were represented as: control group (n=8, ANOVA, $F_{7, 56} = 151$, $P = 0.0000$), low thermal stress group (13°C) (n=8, ANOVA, $F_{7, 56} = 174$, $P = 0.0000$), and high thermal stress group (37°C) (n=8, ANOVA, $F_{7, 56} = 141$, $P = 0.0000$, Fig 14).

Within the control and high thermal stress groups, the TMs strain exhibited the highest expression levels of both HSP90 and HSP70 genes, followed by WRs, WKs, MKs, USs, SPs, IBDs, and CMs, in that order. However, in the low thermal stress group, the IBDs strain showed the highest upregulation of HSP90 and HSP70 genes, followed by CMs, MKS, USs, SPs, WRs, WKs, and TMs strains, respectively. Pairwise multiple comparisons confirmed highly significant differences between the strains in all groups for both HSP90 and HSP70 genes. Additionally, the expression of the HSP90 gene was considerably higher in the high thermal stress groupcompared to the low thermal stress group. Similarly, a greater upregulation was observed in the expression of the HSP70 gene in the high thermal stress group compared to the low thermal stress group.

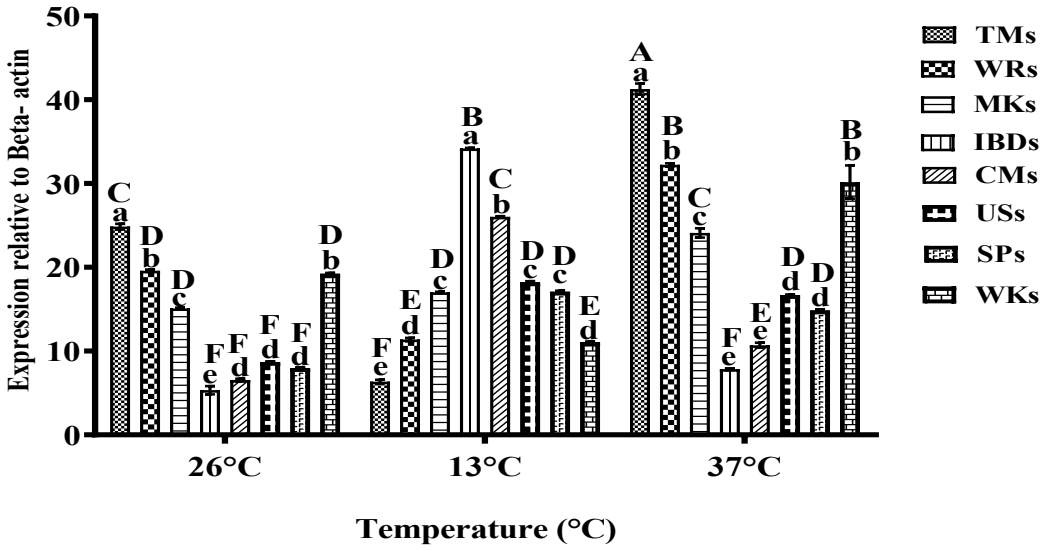


Fig. 17. Expression of HSP90 gene in the liver of different strains of *Labeorohita* under acclimation (26°C), CTMax (37°C), and CTMin (13°C) temperatures. Error bars indicate average ± SEM values of all the strains in three different groups. The lower-case letters showing significant difference among the strains at specific temperature ($P<0.05$) while the uppercase letters show the difference within the strains among different temperatures.

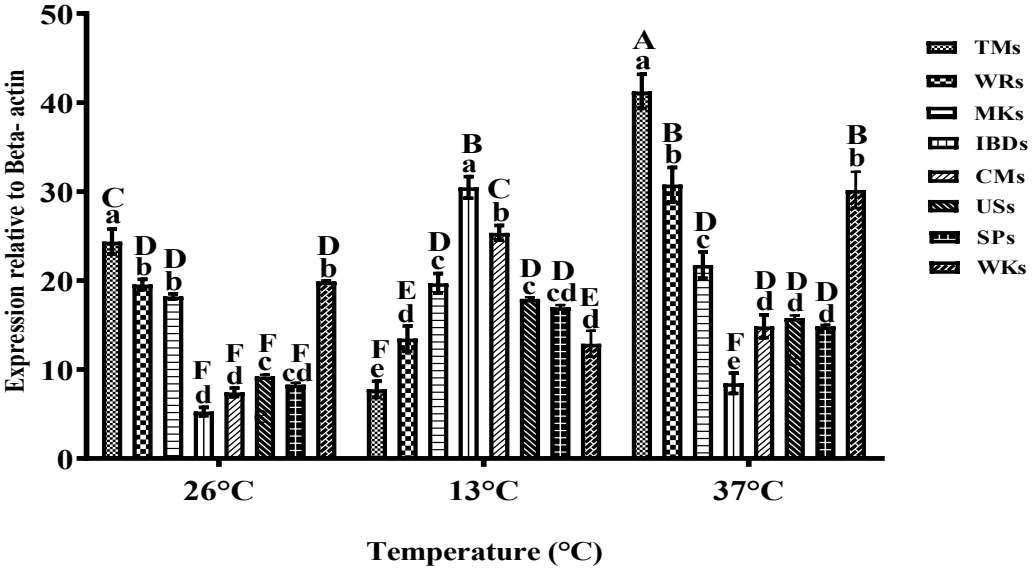


Fig. 18. Expression of HSP70 gene in the liver of different strains of *Labeorohita* under acclimation (26°C), CTMax (37°C), and CTMin (13°C) temperatures. Error bars indicate average ± SEM values of all the strains in three different groups. The lower-case letters showing significant difference among the

strains at specific temperature ($P<0.05$) while the uppercase letters show the difference within the strains among different temperatures.

Challenge test

Among the eight strains of *L.rohita* examined, varying patterns of survival probabilities were observed. The IBDs, MKs, and SPs groups exhibited a consistent decline in survival probability over time, as evidenced by their survival curves gradually decreasing from 1.0. The median survival time for these groups was estimated to be 10 days, with the associated 95% confidence interval suggesting variability in the upper limit.

The CMs group displayed a steep decline in survival probability, with all observations experiencing an event. The median survival time for CMs was estimated to be 3 days, with a 95% confidence interval ranging from 2 to a level of uncertainty in the upper limit. The TMs and WRs groups, characterized by a limited number of events, showed a relatively slower decrease in survival probability compared to other groups. The median survival time for TMs had an associated 95% confidence interval ranging from 10 with a degree of uncertainty in the upper limit. Similarly, the median survival time for WRs had a 95% confidence interval ranging from 9 with some uncertainty in the upper limit.

The analysis revealed varying patterns of survival probabilities over time. Some groups, such as CMs, MKs, and SPs, exhibited a consistent decline in survival probability, while others, like TMs and WRs, showed a relatively slower decrease due to the limited number of events. The results of all strains can be seen in Figure 15.

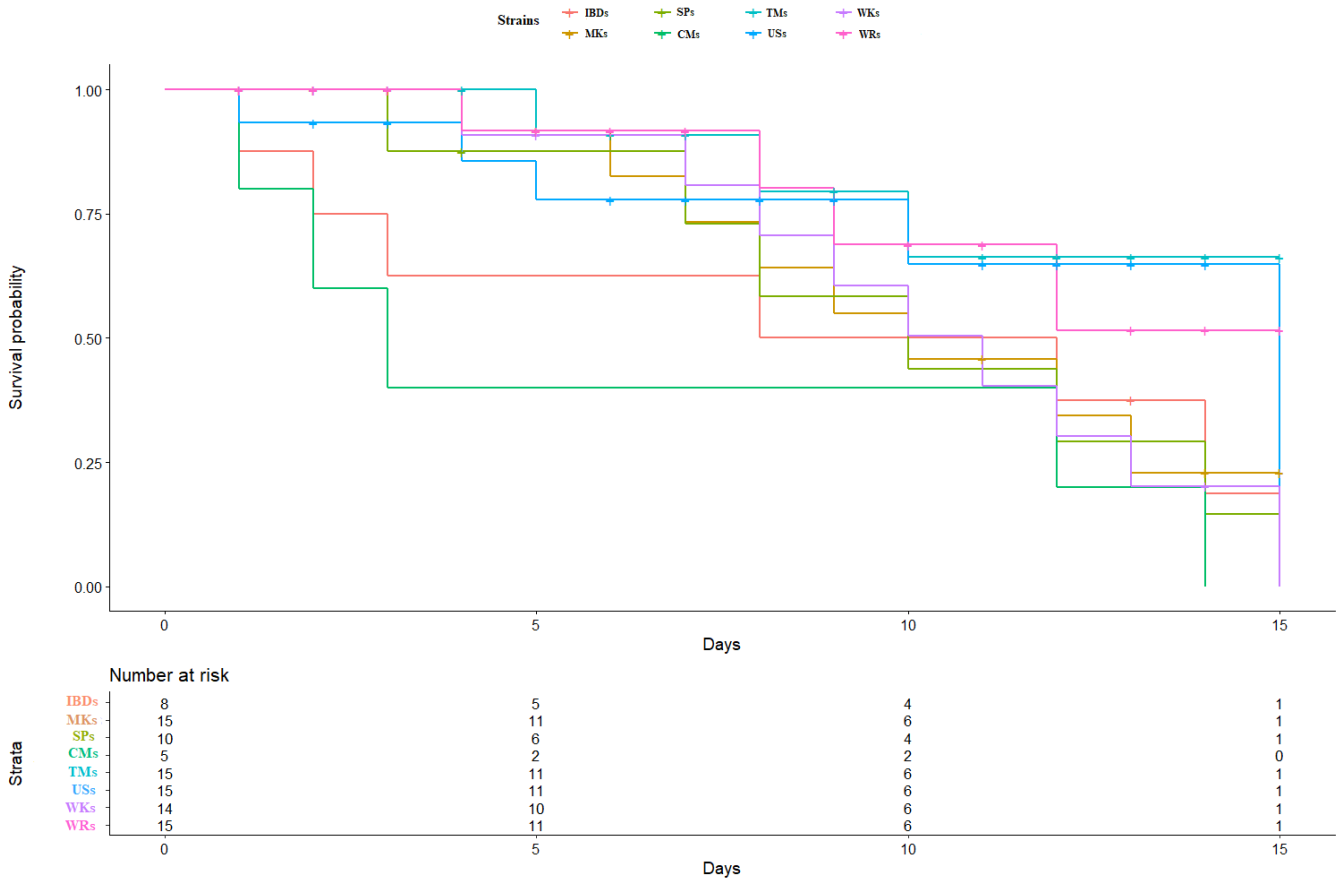


Fig 19: Survival probabilities of all strains through Kaplan-Meier method

Immunological Parameters

The CMs strain displayed a higher AST level (12.00±0.34 UL-1), suggesting a relatively weaker immune response. In contrast, the TMs strain exhibited the lowest AST level (7.25±0.16 UL-1), suggesting better liver health and a relatively stronger immune responsewhile the rest ofthe strains exhibited intermediate responses with WRs and WKs following TMs closely as can be seen in Table 22.Regarding lysozyme levels, the TMs strain showed the highest value (5.57±0.34 µg mL^{-1}), indicative of a more robust immune response. Conversely, the CMs strain exhibited the lowest lysozyme level (2.43±0.12 µg mL^{-1}), suggesting a relatively weaker immune response. Similar trends were observed for total protein levels and IgM, with the TMs strain displaying the highest values (22.32±0.18 mg mL^{-1} and 10.76±0.14 mg mL^{-1}, respectively) and the CMs strain exhibiting the lowest values (14.83±0.32 mg mL-1 and 6.63±0.14 mg mL-1, respectively).

In terms of cellular immune responses, the TMs strain exhibited higher phagocytic activity (53.97±1.09%) and phagocytic index (1.97±0.18), indicating a stronger immune response. In contrast, the CMs strain displayed lower phagocytic activity (37.92±3.21%) and phagocytic index (1.04±0.03), suggesting a relatively weaker immune response. Similarly, the TMs strain demonstrated the highest respiratory burst value (0.74±0.11 OD: 540 nm), indicating a stronger immune response, while the CMs strain exhibited the lowest value (0.24±0.10 OD: 540 nm), suggesting a relatively weaker immune response.

Table 22: Immunological parameters ofall strains of *Labeorohita* after exposure to *Aeromonas hydrophila*

Parameters	TMs	WRs	MKs	CMs	IBDs	USs	SPs	WKs
AST (U/L)	7.25 ± 0.16^{e}	7.48 ± 0.34^{e}	9.33 ± 0.63^{d}	12.00 ± 0.34^{a}	11.10 ± 0.45^{b}	10.01 ± 0.73^{c}	10.32 ± 0.65^{c}	7.52 ± 0.25^{e}
Lysozyme (µg/ mL)	5.57 ± 0.34^{d}	5.00 ± 0.21^{c}	4.32 ± 0.43^{bc}	2.43 ± 0.12^{a}	2.67 ± 0.15^{a}	3.11 ± 0.41^{b}	3.04 ± 0.17^{b}	4.97 ± 0.31^{c}
Total protein (mg/mL)	22.32 ± 0.18^{f}	20.98 ± 0.41^{e}	19.78 ± 0.12^{d}	14.83 ± 0.32^{a}	15.31 ± 0.16^{b}	16.91 ± 0.13^{c}	16.12 ± 0.31^{bc}	20.67 ± 0.13^{de}
IgM (mg/ mL)	10.76 ± 0.14^{e}	9.69 ± 0.08^{d}	8.87 ± 0.16^{c}	6.63 ± 0.14^{a}	6.89 ± 0.11^{a}	7.51 ± 0.16^{b}	7.20 ± 0.14^{b}	9.11 ± 0.17^{d}
Phagocytic activity%	53.97 ± 1.09^{f}	50.62 ± 2.14^{e}	45.96 ± 2.43^{d}	37.92 ± 3.21^{a}	38.43 ± 3.12^{b}	40.12 ± 1.19^{c}	39.99 ± 3.34^{c}	50.10 ± 2.12^{e}
Phagocytic index	1.97 ± 0.18^{d}	1.82 ± 0.11^{c}	1.68 ± 0.07^{b}	1.04 ± 0.03^{a}	1.12 ± 0.05^{a}	1.52 ± 0.06^{ab}	1.48 ± 0.04^{ab}	1.80 ± 0.10^{c}
Respiratory burst (OD: 540 nm)	0.74 ± 0.11^{d}	0.68 ± 0.09^{c}	0.46 ± 0.07^{b}	0.24 ± 0.10^{a}	0.27 ± 0.04^{a}	0.38 ± 0.13^{ab}	0.35 ± 0.06^{ab}	0.65 ± 0.07^{c}

Data are presented as mean ± SEM (n=15). Means sharing similar letters within the row are not significantly different from each other ($P > 0.05$). (ANOVA followed by LSD test).

Hematological Parameters

Comparative hematological analysis of eight strains of *L. rohita* (TMs, WRs, MKs, CMs, IBDs, USs, SPs, and WKs) exposed to *Aeromonas hydrophila* revealed strain-specific variations in parameters such as hemoglobin levels, red and white blood cell counts, platelet counts, hematocrit/packed cell volume, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, red cell distribution width, and mean platelet volume. The TMs strain exhibited higher hemoglobin and red blood cell counts, lower white blood cell count, and stronger immune response. The CMs strain had lower hemoglobin, higher white blood cell count, and lower neutrophil percentage. Platelet count, hematocrit/packed cell volume, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, red cell distribution width, and mean platelet volume varied among the strains where TMs performed better and CMs had the lowest performance. The results can be seen in Table 23.

Table 23: Hematological indices of all strains after exposure to *Aeromonas hydrophila*

	TMs	WRs	MKs	CMs	IBDs	USs	SPs	WKs
Hb g/dl	10.2±0.30^{e}	8.2±0.11^{c}	8.02±0.09^{c}	6.5±0.19^{a}	6.7±0.17^{a}	7.5±0.21^{b}	7.14±0.23^{b}	9.6±0.6^{d}
RBC million/mm^3	2.36±0.40^{d}	1.97±0.60^{c}	1.74±0.20^{b}	1.41±0.28^{a}	1.43±0.15^{a}	1.64±0.11^{ab}	1.62±0.14^{ab}	1.91±0.5^{c}
WBC million/mm^3	1,29,600±65^{a}	1,39,100±60^{b}	1,40,100±75^{bc}	1,50,300±70^{d}	1,45,400±55^{c}	1,41,000±80^{bc}	1,42,100±70^{bc}	1,39,800±900^{b}
Differential Count in %								
Neutrophils	1±0.5^{b}	0.9±0.6^{b}	0.8±0.4^{b}	0.4±0.1^{a}	0.5±0.4^{a}	0.7±0.1^{ab}	0.7±0.2^{ab}	0.9±0.2^{b}
Lymphocytes	96±3.0^{a}	98±6.0^{b}	98±7.0^{b}	106±6.0^{c}	105±7.0^{c}	103±4.0^{c}	101±7.0^{c}	98±4.0^{b}
Eosinophils	1±0.4^{b}	0.3±0.1^{a}	0.2±0.5^{a}	0	0	0.1±0.3^{a}	0.1±0.4^{a}	0.4±0.2^{a}
Monocytes	2±0.1^{a}	2.2±0.5^{a}	2.2±0.3^{a}	2.4±0.5^{b}	2.4±0.4^{b}	2.3±0.1^{b}	2.3±0.1^{b}	2.1±0.2^{a}
Basophils	0	0.1±0.01^{a}	0.2±0.01^{a}	0	0	0.1±0.01^{a}	0.1±0.01^{a}	0.2±0.01^{a}
Platelet Count /mm^3	3,27,100±50^{a}	78,900±800^{c}	75,200±400^{c}	1,02,500±70^{b}	100000±300^{b}	100100±600^{b}	102000±650^{b}	53,700±350^{c}
Hematocrit/Packed Cell Volume Vol%	33.3±5.2^{f}	18.5±4.0^{e}	16.3±3.1^{d}	13.29±2.2^{b}	13.78±2.7^{b}	14.4±2.3^{c}	14.6±2.0^{c}	10.3±1.9^{a}
MCV fl	134±11^{e}	130±18^{d}	124±12^{c}	107±19^{a}	109±17^{a}	117±14^{b}	111±15^{ab}	126±12^{c}
MCH pg	44.7±6.0^{d}	44.1±5.0^{d}	43.2±5.5^{c}	40.1±6.0^{a}	40.3±4.0^{a}	41.5±5.0^{b}	41.2±4.7^{b}	44.3±7.0^{d}
MCHC gm/L	34.9±3.2^{e}	35.7±5.3^{e}	37.8±3.5^{c}	42.7±6.4^{a}	42.5±4.6^{a}	41.5±4.8^{b}	41.8±4.0^{b}	36.4±4.6^{d}
RDW %	11.3±1.9^{d}	11.6±1.9^{d}	11.3±1.0^{d}	20.5±2.4^{a}	19.4±2.0^{b}	18.4±2.3^{c}	18.6±1.9^{c}	11.7±1.8^{d}
MPV	5.8±1.2^{a}	5.5±1.0^{a}	5.7±3.0^{a}	6.9±2.3^{c}	6.6±1.9^{bc}	5.8±1.5^{a}	6.1±1.7^{b}	5.4±2.3^{a}

One-way ANOVA followed by LSD Post Hoc test shows a pairwise comparison between strains. Alphabets on mean values are significantly showing difference ($P < 0.05$) between stocks.

Expression of Immunity related genes

The examination of C9 gene expression in various stocks of *Labeorohita* fingerlings following exposure to *Staphylococcus aureus* unveiled distinct temporal patterns (Fig. 20-22). Statistical analysis revealed significant differences among strains with respect to the post challenge temporal expressions of immunity related genes (C9: n = 9, ANOVA, F7, 64 = 680, p = 0.000 (Fig. 20); Transferrin: n = 9, ANOVA, F7, 64 = 803.51, p = 0.000 (Fig. 21)) Hepcidin: n = 9, ANOVA, F7, 64 = 710.32, p = 0.000 (Fig. 22). Noteworthy trends were observed, with C9 expression peaking significantly at 56 hours post-exposure, exhibiting a substantial increase compared to the baseline level at 0 hours. This peak was succeeded by a slight decline at 112 hours, although the expression remained notably higher than at the initial stage. By 168 hours post-challenge, there was a further decrease in expression, although remaining above the baseline. Similar temporal patterns were observed for the expression of transferrin and hepcidin genes, with peak expression also occurring at 56 hours post-challenge, followed by a decline at 112 hours and 168 hours. Notably, among the three genes investigated, C9 demonstrated the highest expression levels across all durations.

Furthermore, post hoc analysis revealed that TMs consistently displayed the highest expression at all time points, with MKs, WRs, and WKs closely following. Intermediate expression levels were observed in SPs and USs strains, while IBDs and CMs strains exhibited the lowest expression levels across all genes and time points.

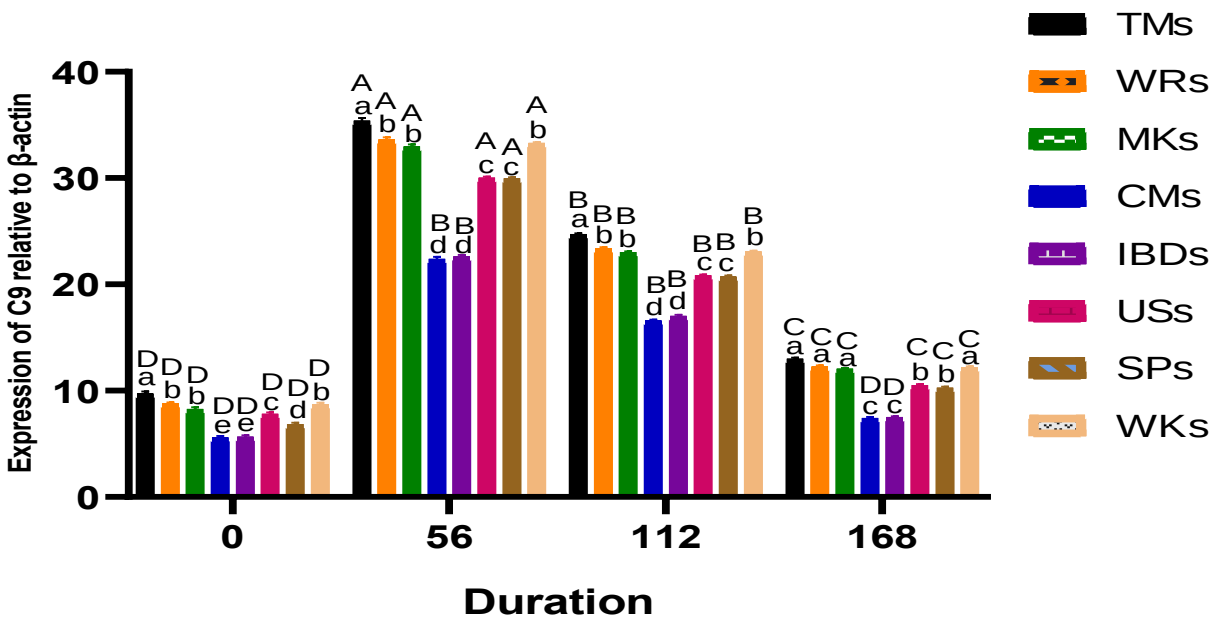


Fig. 20. C9 gene expression in different stocks of *L. rohita* fingerlings after 0, 56, 112, and 168 h post-challenge with *S. aureus.* The bar shows the values as average ± SD.Two-way ANOVA followed by LSD post hoc test show multiple comparison among the strains. The lower-case letters showing significant difference among the strains at specific time (P<0.05) while the uppercase letters show the temporal difference in expression among strains.

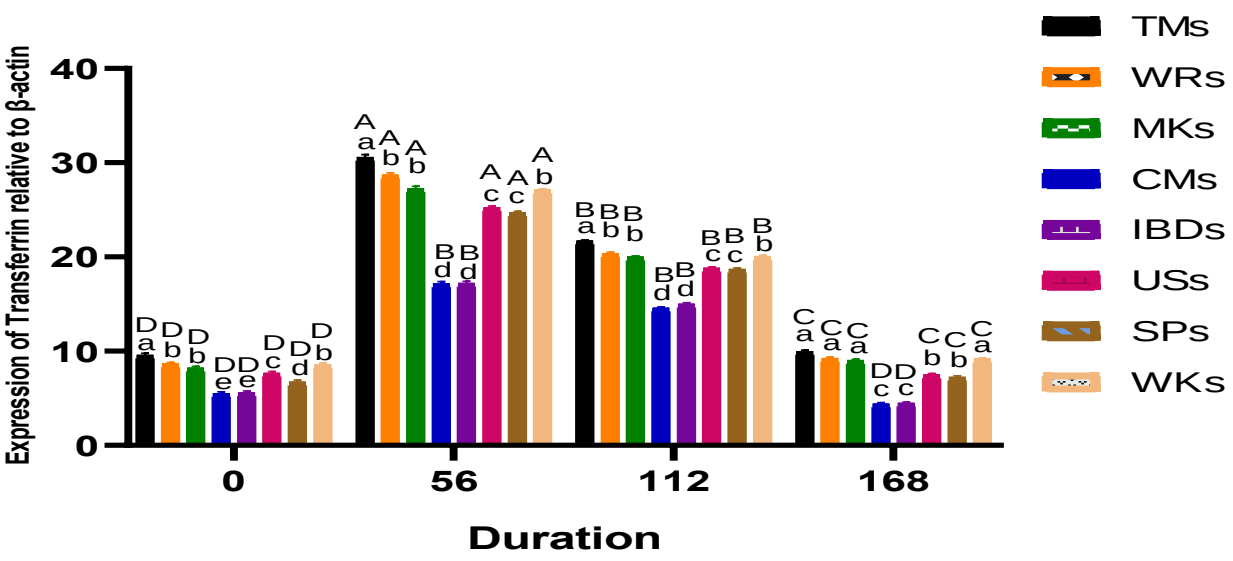


Fig. 21. Transferrin gene expression in different stocks of *L. rohita* fingerlings after 0, 56, 112, and 168 h post-challenge with *A. hydrophila*. The bar shows the values as average ± SD. Two-way ANOVA followed by LSD post hoc test show multiple comparison among the strains. The lower-case letters showing significant difference among the strains at specific time (P<0.05) while the uppercase letters show the temporal difference in expression among strains.

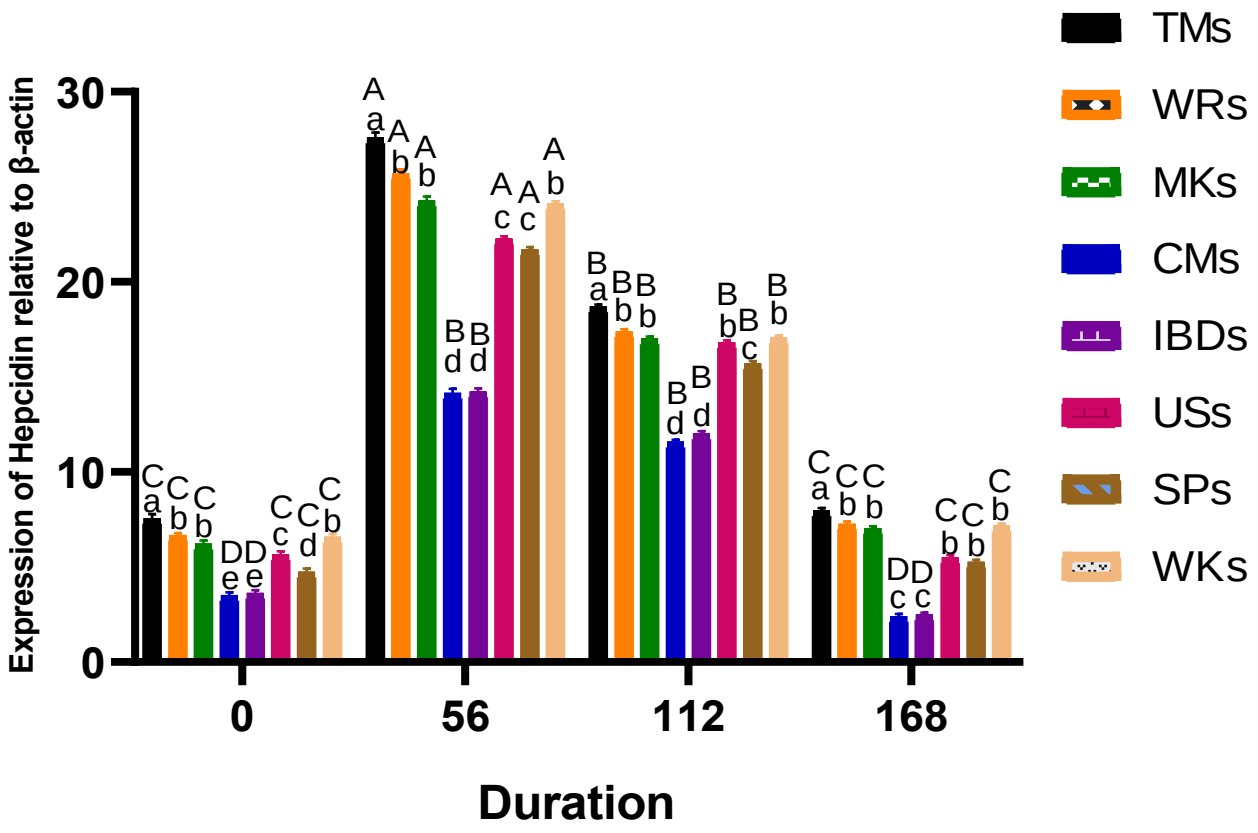


Fig. 22. Hepcidin gene expression in different stocks of *L. rohita* fingerlings after 0, 56, 112, and 168 h post-challenge with *S. aureus.* The bar shows the values as average ± SD.Two-way ANOVA followed by LSD post hoc test show multiple comparison among the strains. The lower-case letters showing significant difference among the strains at specific time (P<0.05) while the uppercase letters show the temporal difference in expression among strains.

DISCUSSION

Studying the differences and genetic distances among populations of a species is crucial for creating genetically superior strains through hatchery production (Rana et al., 2004; Baisvar et al., 2019). In this research, the focus was on examining the genetic diversity among *Labeorohita* fish collected from eight different sites. The goal was to understand the genetic variations and distances among these strains. The selected hatcheries for this study were Tawakal Fish Hatchery, Mian Chunnu Fish Hatchery, Charbanda Fish Hatchery, River Ravi, River Kabul, Upper Sindh Govt. Fish Hatchery, Sherabad Carp Hatchery and Training Center, and Rawal Fish Hatchery. Importantly, these chosen hatcheries are in geographically isolated areas. Tawakkalhatchery is situated in the Muzaffargarh district of the Punjab province, precisely located at the geographical center of Pakistan. The selected Miachannufish hatchery, district Multan is in Punjab province. Charbandafish hatchery is in district Mardan, Khyber Pakhtunkhwa. However, Rawal town fish seed hatchery is situated in Islamabad which has a humid subtropical climate.Sherabad, located near warsakroad in Peshawar. Upper Sindh, like much of the province, generally experiences a hot desert climate.

The region may also witness some cooler nights during the winter season.The river Ravi traverses regions of both India and Pakistan, and the local climate is significantly influenced by its geographical setting. The area is generally characterized as subtropical, featuring hot summers and relatively cool winters. Summertime highs frequently surpass 40 degrees Celsius (104 degrees Fahrenheit), resulting in a warm and occasionally intense atmosphere. Winters, on the other hand, are relatively mild, with daytime temperatures ranging from cool to moderate, typically between 10 to 20 degrees Celsius (50 to 68 degrees Fahrenheit). Additionally, the region encounters monsoon rains during the summer months, contributing to the overall climatic dynamics.Meanwhile, the Kabul River, coursing through Afghanistan and Pakistan, experiences a diverse climate, ranging from arid and semi-arid conditions to more temperate climates in certain areas. Lower altitude summers can be quite hot, with highs of around 30 degrees Celsius (86 degrees Fahrenheit). while winters tend to be cooler, particularly in higher elevations, where snowfall is not uncommon during the winter months, especially in the mountainous terrain.

Besides climatic condition these Hatcheries also have different histories in managements, husbandry practices and induced spawning program. In Pakistan most of Government fish Hatcheries including

these, are using conventional breeding program with a limited number of broodstock, therefore facing the inbreeding depression or loss of genetic vigor which appeared in the present results in some strains. The Tawakkal Fish Hatchery has the higher number of broodstock thus showing significantly improved growth, innate immunity, disease resistance and blood profile etc. in contrast to other Hatcheries stocks, showing how breeding programmes and management techniques affect fish phenotypic and genotypic characteristics.

Microsatellite analysis showed significant genetic variation among *L. rohita* strains, with diverse Ho and He values. TMs showed the highest genetic variability, while TKs exhibited the lowest. Deviations from Hardy-Weinberg Equilibrium were observed in 12 tests, impacting various loci across strains. Pairwise comparisons highlighted considerable genetic differentiation, supported by moderate to low microsatellite FST values. Notably, WKs and WRs displayed the highest gene flow, contrasting with CMs and MKs, which exhibited the highest dissimilarity and lowest gene flow.

The speedy evolution observed in mitochondrial genes enables discrimination among populations, even with a limited sample size (Sharma et al., 2019). A 680bp segment of the COI gene was amplified and subjected to sequencing. The study outcomes revealed substantial genetic variability and a significant level of genetic differentiation among populations. These results align with Sharma et al. (2018) findings, highlighting the ability to determine a meaningful correlation between geographical distances and genetic differentiation using the chosen mitochondrial marker.

The investigation involved pairwise comparisons between each strain, revealing significant genetic differences (Table 3). The analysis of genetic distance, based on the CO1 gene, showed varying degrees of gene flow. Notably, MKs-TKs, WRs-CMs, WRs-MKs, WRs-TMs, and MKs-WKs exhibited the highest gene flow (FST = 0.001), while the minimum gene flow occurred between WRs and USs (0.068).

The UPGMA dendrogram, presented in Fig 5 A and B and utilizing Nei‘s genetic distance (1972), showcased the genetic relatedness among all strains. The dendrogram formed two major clusters. In the first sub-cluster, only the USs strain was present, while the second sub-cluster included TMs and MKs. The second major cluster was further divided into two clusters, with WRs and WKs in one and CMs, TKs, and SPs in the other. This clustering pattern was consistent across both microsatellite and mtDNA markers, emphasizing the genetic distinctiveness among the various strains of *L. rohita* from different

hatcheries. The results showed that *L. rohita*populations from different locationscanbeconsideredasdistinctevolutionaryunits.Becauseofthis,thesestrainscanbetakenasdistinctunits of management during the implementation of conservation methods. The correlation in the geneticvariation and the geological distance among strains showed that environmental conditions also accountforgeneticvariabilitiesin*L.rohita*strainsandmayprovetheprimefactorinaffectingthegeneticstructure of a population. These findings are also like the results reported by Sharma et al. (2018)regardingChocolate mahseer based onmitochondrial DNAmarkers.

Ouroutcomesshowedthatthegeneticdiversitywasloweramongpopulationsascompared to that reported by Behera et al. (2018) among the wild populations of *L. rohita*from differentrivers.Thismaybebecause a restrictednumberofindividualsarekeptathatcheriessampledfromthe natural environment andtherepeated breeding eventsamong them and the restocking programs carried out by various hatcheries.

After assessing genetic diversity, the subsequent step involved evaluating the growth performance and immunity of advanced fry and fingerlings. Since both factors are influenced by genetic makeup, environmental factors, and their interaction advanced fry from all stocks were initially raised in controlled conditions within glass aquaria. Subsequently, the experiment was repeated at the fingerling stage for all stocks, with careful consideration to minimize environmental effects. During this stage, the fish were tagged and reared in a communal pond. The results demonstrated discernible differences in both growth performance and immune response among the stocks. TMs and WRs displayed superior growth and survival, closely followed by MKs, while IBDs and CMs exhibited lower growth and survival rates. These findings align with a study conducted by Reddy et al. (2002), which investigated the growth and survival of six populations of *L. rohita* carps in earthen ponds under both monoculture and polyculture systems. These populations included one domesticated stock and five wild stocks. The research revealed noteworthy differences in harvest weight and survival rates among the strains, suggesting that selective breeding could effectively enhance these traits, The higher growth rate in TMs was attributed solely to their superior genetic makeup. These findings align with Redyy et al. (2002) observations, underscoring the significant impact of selective breeding on improving growth traits.

In addition, various parameters, including immune response, thermal tolerance, and the expression of HSP genes (HSP70 and HSP90), were examined. Notably, TMs exhibited a heightened response compared to the other strains. Given identical environmental conditions, this disparity may stem from

superior management, adept husbandry practices, induced spawning programs, and a more extensive array of brood stocks in TMs in comparison to other strains. These factors collectively contribute to a greater genetic vigor in TMs, underscoring the impact of management and breeding programs on both genetic and non-genetic traits within this strain.

The ongoing investigation has uncovered notable differences in the innate immune response among all the strains. These variations may arise from genetic factors (Fjalstad et al., 2003), biotic and abiotic agents (Jawad et al., 2004), and the impact of management (Davis et al., 2002). Additionally, these discrepancies may manifest within species and across species, depending on the specific aquaculture systems in place (Barcellos et al., 2009). The present study involved the assessment of various crucial immunological indicators in fish blood, including total serum protein contents, total IgM, Lysozyme activity, respiratory burst, and phagocytic activity.

In the present investigation, fish blood was analyzed to determine total protein contents and total IgM. Different strains exhibited varying levels of total serum protein, with TMs reaching the highest levels and CMs displaying the lowest. The differences in total protein contents among all strains were statistically significant ($P<0.05$). The elevated values observed in this study were consistent with findings by Wijendra and Pathiratne (2007), while the lower values fell within the range reported by Mohapatra et al. (2014) and Pakhira et al. (2015). Similarly, IgM levels mirrored the trend seen in total protein, with TMs showing higher levels compared to other strains, and CMs registering the lowest IgM levels. These results indicated a slight correlation between IgM levels in the current study and previous research, such as that conducted by Mohapatra et al. (2012).

The lower IgM and total protein levels in CMs could be attributed to stress, infections (Rehulka, 1993), or certain chronic diseases (Martin et al., 2010). Numerous studies have noted variations in IgM levels in fish, like other immune parameters, which may be influenced by factors such as size and age (Picchietti et al., 2001). It is suggested that higher levels of total protein, IgM, and other globulins signify a robust immune system in fish, as highlighted by Wiegertjes et al. (1996).

Lysozymes play a crucial role in eradicating both gram-positive and gram-negative bacteria through the degradation of the peptidoglycan layer (Yano, 1996). They are widely recognized as indicators of a nonspecific immune system (Saurab and Sahoo, 2008). In the current research, TMs exhibited relatively higher lysozyme activity, while CMs displayed the lowest activity. These results are consistent with

research by Reddy et al. (2002) that examined the growth and survival of six populations of *L.rohita*carps in earthen ponds under monoculture and polyculture systems—one domesticated stock and five wild stocks. The results of the study showed statistically significant variations in harvest weight and survival rates amongst strains, indicating that these two characteristics might be successfully improved through selective breeding. Our results align with their studies and fall within the acceptable range.

The reduced lysozyme activity in CMs suggests a weakened disease resistance in this strain. Conversely, the heightened lysozyme activity in TMs indicates an elevated presence of different humeral agents contributing to the defense system of this strain, thereby enhancing its protection against pathogens and various diseases.

Blood cells, including neutrophils, monocytes, and lymphocytes, play a crucial role in organic defense and are significant (Martins et al., 2008; Martins et al., 2009). In our research, we observed notable differences in phagocytic activity among the strains, specifically in the percentage of macrophages with ingested bacteria. TMs exhibited the highest percentage of macrophages, while CMs displayed the lowest. Various studies on fish phagocytic activity have been conducted, and our results closely resembled those reported by Harikrishnan et al. (2011) and Solis et al. (2007). Cai et al. (2004) documented a phagocytic activity of 66% in three Tilapia species. The Phagocytic Index (PI) also reflected similar trends, with TMs exhibiting the highest PI value, followed by WRs, WKs, and MKs, while other strains showed intermediate results, with CMs having the lowest PI among all the strains.

Upon encountering invading microorganisms, macrophages initiate antimicrobial actions by employing reactive oxygen species such as H2O2, peroxides, and lysozymes (Secombes and Fletcher, 1992). Phagocytes generate respiratory bursts to break down the invading organisms (Jian and Wu, 2003). According to Ellis (2001), peroxides are produced during respiratory burst activity and function as bactericidal agents. In the current findings, it was observed that TMs exhibited the highest level of respiratory burst activity, while CMs displayed the lowest. The lysozyme and respiratory burst activity complemented each other in the present study, indicating an enhanced resistance to disease in TMs. Phagocytosis and respiratory burst are believed to be interrelated and constitute a crucial component of the non-specific defense system in fish (Jian and Wu, 2003).

Adapting to climate changes, thermal tolerance emerges as the most pivotal factor enabling animal populations to navigate variations in temperature (Portner and Knust, 2007). To assess this, a thermal tolerance assay was conducted on various strains of *Labeorohita* acclimated to 26°C. Every strain's thermal tolerance was assessed for both the lower and upper thermal limits (CTMin and CTMax). For every strain, different CTMax and CTMin values were found, most likely due to genetic or environmental influences (Imsland et al., 2003; Nakajima et al., 2009). In this study, TMs exhibited higher CTMax and CTMin, while IBDs displayed the lowest CTMax and CTMin values among all strains. Our findings align closely with studies on thermal tolerance in different fish species, including early fingerlings of common carp (Chatterjee et al., 2004), Catfish (*Horabagrusbrachysoma*) (Dalvi et al., 2009), and fingerlings of Indian Major Carps (Das et al., 2004).

Drawing from CTMax and CTMin data, it can be inferred that adaptations to temperature variations are vital for fish and are dependent on the acclimation temperature (Beitinger et al., 2000). Consequently, the present study affirms the existence of intraspecific variations in thermal tolerance.

Each strain exhibited heightened thermal tolerance, signifying that an increased CTMax corresponds to enhanced tolerance to heat, while a higher CTMin indicates a reduction in heat tolerance. Therefore, it is deduced that the observed rise in CTMax and CTMin values correlates with increasing and decreasing temperatures, respectively. Studies by Bennett and Beitinger (1997) and Dalvi et al. (2009) underscore the substantial influence of acclimation temperature on the thermal tolerance levels of fish.

Thermal tolerance polygons offer valuable insights into the thermal physiology, ecological preferences, and distribution strategies of fish species (Das et al., 2004; Dülger et al., 2012). In our study, we applied thermal polygon analysis to investigate eight strains of *Labeorohita* (TMs, WRs, CMs, MKs, IBDs, USs, SPs, and WKs) and examined their thermal tolerance zones across four fixed temperatures (22–34 °C). Our results revealed significant differences in total, intrinsic, and acquired thermal tolerance zones among the strains. TMs exhibited the widest thermal tolerance range, with a total polygon area of 298.5°C, indicating their ability to withstand diverse thermal conditions. WRs displayed a narrower thermal range (292.11°C), suggesting a more specialized thermal niche. CMs exhibited a unique thermal pattern with a broad total polygon area (297.18°C), where the slope indicated decreasing thermal tolerance with higher acclimation temperatures. MKs displayed a balanced thermal range (294.98°C), with similar upper and lower areas. IBDs showed a moderate thermal range (292.59°C), and USs and

SPs displayed balanced thermal ranges with slight increases in thermal tolerance as acclimation temperature rose. The high R2 values (0.922 to 0.981) across all strains signify a robust correlation between acclimation temperature and thermal tolerance, validating the reliability of our polygon analysis. These findings contribute to a comprehensive understanding of the thermal adaptations of different *L. rohita*strains. Similar observations were made by other researchers that indicate differences in thermal tolerance (Chatterjee et al., 2004; Das et al., 2004). In this study, it was noted that the thermal polygons of all four populations under investigation surpass those documented by Chatterjee et al. (2004) for *Labeorohita* within the acclimation range of 25–35 °C. However, they are notably smaller than the extensive 744.8°C2 reported by Das et al. (2004) for *L. rohita*over a broader acclimation temperature span of 12–40°C. The variability in these findings could be attributed to distinctions in acclimation temperatures. The total thermal polygon area is intricately linked to the specific acclimation temperatures employed in the experiment, as highlighted in previous research (Das et al., 2005). Consequently, it can be proposed that TMs may be the most suitable strain for warm water culture, while IBDs may be better suited for semi-cold-water culture in Pakistan.

Heat Shock Proteins (HSPs) are highly conserved proteins produced by organisms in response to stress conditions, playing a crucial role in stabilizing homeostasis and restoring degraded cell components (Valenzuela-Castillo et al., 2019). In this study, two types of HSP genes, namely HSP90 and HSP70, were investigated in various strains of *L. rohita* acclimated to a temperature of 26°C. These strains were subjected to both low (13°C) and high (37°C) thermal stress. HSP genes are essential and extensively studied proteins that function as molecular chaperones, assisting in processes such as protein folding, membrane translocation, degradation of misfolded proteins, and other regulatory functions (Mayer and Bukau, 2005; Pearl and Prodromou, 2006; Multhoff, 2007; Ming et al., 2010). Their expression was evaluated relative to β-actin. In terms of relative expression across all strains and groups, TMs exhibited high upregulation of both genes in the control and high thermal stress groups, followed by WRs, WKs, MKs, USs, SPs, IBDs, and CMs, respectively. Notably, IBDs displayed high expression in the low thermal stress group. Upon multiple comparisons between strains at all temperatures for both HSP90 and HSP70, TMs consistently demonstrated the highest expression, followed by WRs, WKs, MKs, USs, SPs, IBDs, and CMs, respectively.

Like our investigation, several other researchers have endeavored to examine the expression of HSP genes in response to various stressors, including temperature stress, toxic chemicals, and more (Fangue et al., 2006; Rosic et al., 2011; Stitt et al., 2014; Eid et al., 2016; Kelly et al., 2018). For instance, Eid et al. (2016) subjected Snakehead fish to two different temperatures (16°C and 32°C) and explored the HSP70 gene expression in various tissues of the body. Their findings indicated that HSP70 was markedly upregulated at 32°C compared to 16°C in different tissues. The observed trend in gene expression in their study aligns with our results, where we identified higher expression of both HSP90 and HSP70 genes under high thermal stress compared to low thermal stress.

Our findings reveal that TMs exhibited a robust heat shock response to high thermal stress, whereas RWL-s demonstrated a strong response to low thermal stress. The results suggest that TWK-s could maintain physiological conditions under a wide range of variations in warm water temperature and might survive even at relatively high temperature ranges. Similarly, IBDs showed tolerance to low-temperature variations. Furthermore, through strain-to-strain and groupwise comparisons, it was observed that the HSP70 gene exhibited higher upregulation compared to the HSP90 gene. The significant upregulation of HSP70 genes indicates superior thermal tolerance in different cells (Li et al., 1992).

The present investigation also disclosed that TMs demonstrated the greatest likelihood of survival, as determined through the Kaplan-Meier method. Subsequently, WRs, WKs, and MKs exhibited progressively lower survival probabilities following a pathogenic challenge. This heightened probability of survival implies an elevated level of disease resistance compared to other strains. The notable improvement in survival rates could be attributed to the enhanced stimulation and activation of the innate immune system, as expounded by Puddu et al. (2014). Similar findings were documented in diverse populations of Atlantic salmon *(Salmo salar)* (Moen et al., 2009), Japanese indigenous and domesticated Eurasian common carp (Ito et al., 2014), and common carps *(C. carpio)* (Tadmor-Levi et al., 2017), illustrating variability in disease resistance against distinct pathogens among different populations.

In the pursuit of understanding the molecular mechanisms governing growth and immunity, our gene expression study unveiled elevated expression levels of growth and immunity genes in the TMs strain, followed by WRs, WKs, and MKs. This outcome corroborates our previous findings on growth

performance obtained from rearing all populations under controlled and semi-intensive culture conditions, hinting at the potential utilization of these strains for breeding a rapidly growing lineage of *L. rohita*. The positive association between growth performance and the expression of growth genes aligns with Pérez-Sánchez et al.'s (1995) investigation. IGF II, a pivotal factor in vertebrate growth regulation, including fish, exhibited higher expression levels in the liver compared to muscle. This discrepancy is attributed to its dependence on growth hormone synthesis occurring in the carp's liver (Margaret et al., 2002; Peterson et al., 2004; Kumar et al., 2018; Reinecke et al., 2005; Picha et al., 2008; Fox et al., 2010; Ghelichpour et al., 2019; Beckman and Dickoff, 1998).

Moreover, mRNA expression of preproghrelin was detected in the muscle and liver of fish from all strains. Like IGF II, preproghrelin exhibited higher expression in the TMs strain, supporting its association with superior growth performance. The upregulation of preproghrelin triggered an increase in GHSR levels, contributing to optimal fish growth. GHSR, an important gene regulating compensatory growth, appetite, feeding, and energy homeostasis, showed higher expression in the liver compared to muscle, emphasizing its role in these physiological processes (Bélanger et al., 2002; Unniappan et al., 2004; Jönsson et al., 2007; Jiwyam, 2010; Dar et al., 2018).

Our findings demonstrated that the TMs stocks exhibited elevated expression of NKEF in both the liver and muscle, surpassing the expression levels observed in WRs, WKs, and MKs. In contrast, the IBDs and CMs stocks displayed notably lower expression, signaling a diminished ability to combat diseases compared to other hatcheries. USs and SPs showed intermediate expressions in this regard.

The NKEF gene possesses the potential to augment the cytotoxic activity of natural killer cells during conditions such as tumor growth or uncontrolled cell division, as indicated by previous studies (Sauri et al., 1995; Kim et al., 2011; Das et al., 2011). Additionally, it functions as an antioxidant, shielding cellular molecules from superoxide radicals. In our study,in contrast to the fish's muscles, we found that the liver had a greater expression level of NKEF. This disparity can be attributed to the diverse functions performed by the liver, including detoxification and antioxidation, aligning with findings reported by Dong et al. (2007) and Huang et al. (2009). The elevated expression in the liver underscores the crucial role of NKEF in innate immunity (Kim et al., 2011).

We examined the mRNA expression of TNF-α in several stocks of *L. rohita* after pathogen exposure, like the NKEF gene expression study. Consistent with previous findings, TNF-α gene expression demonstrated a significant upregulation in TMs, WRs, WKs, and MKs stocks. TNF-α is recognized as a product of innate immunity, playing a crucial role in respiratory bursts, phagocytosis, and the inflammatory process (Sahu and Lambris, 2001). Additionally, TNF-α plays a vital role in adaptive immunity, contributing to the mediation and enhancement of humoral immunity.

Summary of Findings and Conclusions:

The current study systematically evaluated eight genetically distinct strains of *Labeorohita,* one of the most economically important freshwater fish species in South Asia, particularly in Pakistan. The evaluation was conducted across multiple key parameters, including genetic diversity, growth performance, thermal tolerance, immune response, and resistance to disease, with the overarching aim of identifying strains with the greatest potential for genetic improvement and aquaculture enhancement.

Based on advanced genetic markers such as Cytochrome Oxidase I (COI) and microsatellite markers, this research showed substantial genetic diversity among these strains which indicates significant variability within *Labeorohita* population. This type of genetic heterogeneity is vital for adaptation to various environmental changes and long-term sustainability of the species. TMs strain was found to be most promising among those examined since it had the best performance across all major parameters. In this regard TMs compared to others had highest rates of growth, exceptional thermal tolerance as well as robust immune responses besides being notably more resistant to disease than any other strains. These properties imply that there may be advantages in terms of genetics possessed by TMs which could be exploited for enhancing aquaculture output.

When it comes to enhancing the productivity of *Labeorohita* in Pakistan, these findings provide necessary baseline data for the design of a genetic improvement program. Potential for increasing fish production significantly exists by incorporating superior strains such as TMs into selective breeding programs. Furthermore, this research highlights the need for conserving and promoting genetic diversity within aquaculture systems since it is an important determinant of resilience against environmental stressors and outbreaks of diseases which are major issues globally due to climate change and increasing demand for fish.

The study‘s conclusions support that choosing better strains can improve genetic variability significantly through breeding programs aimed at aquaculture expansion. More specifically, it calls for properly constructed breeding programs focusing on economically important traits like growth rate, adaptation abilities and resistance from many pathogens to promote viability and long-term sustainability in aquaculture businesses around Pakistan. In addition, these results have serious implications for fishery policy and management in Pakistan by offering practical recommendations for using fisheries resources wisely in the future.

Achievement of Aims and Objectives:

It is believed that a standard population of *Labeorohita* strains was established for genetic improvement plan by this research work. Not only did the research meet all its goals but also produced useful data that will serve as the basis for a complete breeding strategy aimed at enhancing aquaculture in Pakistan.

To accomplish its first goal, which was to assess genetic diversity among *Labeorohita* stocks, this study utilized advanced molecular techniques such as microsatellite markers and mitochondrial DNA analysis (COI gene). These genetic markers offered a high-definition view of variation within and across studied strains indicating considerable genetic differentiation. The implications of this finding are critical to understanding evolution potential of the species as well as identifying strains with desirable characteristics for selective breeding.

Another objective was realizing the comparison on different cultural systems regarding fry and fingerlings growth performances. Through careful experimentation, the study was able to demonstrate that growth performance varies considerably between strains, with certain strains exhibiting superior growth rates under specific culture conditions. This information is vital when optimizing fish farming practices because they recommend that various strains might fit best into different biomes and breeding styles leading to more selective and effective aquaculture practices.

The third objective of the study was to evaluate how different strains responded to temperature variations and stress. In a series of controlled temperature experiments designed to measure thermal stress tolerance, it was determined that strain TMs had excellent thermal resistance which is very important for global warming and increasing uncertainty in aquatic ecosystems. The capacity to tolerate temperature variations enhances this strain‘s chances of performing well under changing climatic conditions which makes it a good candidate for genetic modification program.

Furthermore, another aim included in this work was to analyze immunity and disease resistance among the various strains; this was done by deliberately inflicting pathogens on fish followed by assessing their immune responses against the pathogens. The results indicated considerable differences existing within the strains‘ immune competences where fish from TMs had improved resistance towards common aquatic

pathogens. This improved body defense mechanism is essential for lowering death numbers in fish farming systems, hence increasing effectiveness and durability of production.

At last, biochemical analyses of fish nutritional value fulfilled the fifth objective which was to compare strain proximate compositions. The study established that there were variations among strains in terms of their proximate composition with some strains having more proteins and lipids which are important factors not only for the growth rates and health of the fish but also determine their market value as well as consumer‘s nutritional quality.

The outcomes of these objectives collectively provide a complete framework for development of an adequate genetic improvement strategy for *Labeorohita* in Pakistan. This research yields a roadmap towards improving genetic stock used in aquaculture by discovering strains possessing superior growth rate, thermal tolerance, immune response and nutritional composition. The data obtained from this study will serve as the basis upon which future breeding programs targeting strains that are more resilient, productive and adaptable to environment should be built hence ensuring sustainable development of aquaculture in Pakistan.

Recommendations for Future Research and Implementation:

Based on this study‘s results, a few crucial suggestions are put forward to guide ongoing research efforts and introduce genetic improvement programs for *Labeorohita* in Pakistan. These measures aim at maximizing potential from superior strains, increasing aquaculture output in general as well as ensuring industry sustainability.

Step 1: Start up Breeding Programs Targeting Superior Strains (e.g. TMs)

Primarily there should be establishment of selective breeding programs focusing on superior strain like TM that have shown excellent performance in terms of growth rate, thermal tolerance and disease resistance. It is necessary to reproduce these beneficial qualities consistently to improve productivity of aquaculture systems throughout the country. Genetic improvement of such strains will assist fish farmers reap improved yields while minimizing resource wastage and lowering vulnerability to environmental stressors thus boosting economic development within the sector.

Step 2: Conduct Further Genetic Mapping and Functional Genomics Studies

For the full utilization of superior strains, there is need for comprehensive genetic mapping and functional genomics studies. The goal of these efforts should be to identify specific genes or genetic regions associated with desirable traits such as accelerated growth rates, enhanced immune response, and disease resistance. By pinpointing the molecular mechanisms conditioning these traits, future research can focus on fine-tuning selective breeding strategies. The potential use of gene-editing technologies like CRISPR/Cas9 could also be explored for direct manipulation of these genetic traits which may lead to increased breeding speed and production of more resilient strains.

Step 3: Establish Long-term Monitoring Programs for Environmental Resilience

Due to the increasing unpredictability of environmental conditions in relation to climate change, it is important to carry out long-term monitoring on selected strains subjected to various environmental stressors including fluctuating water temperatures, salinity levels as well as pathogen exposure. Such action will ensure that superior strains, especially TMs, remain resilient and productive over time.

Step 4: Collaborate with Hatcheries and Stakeholders Nationwide for Selective Breeding Implementation

To succeed in selective breeding programs, there is a need for collaboration between research institutions, hatcheries and other stakeholders in the aquaculture sector. In this way, partnerships with hatcheries across the country can support the continuation of distribution of high-quality genetic strains found in this study. In addition, capacity building initiatives for operators of hatcheries and fish farmers can be started to ensure proper transfer of knowledge that is technical on selective breeding and best practices. This collaborative approach will help scale up the benefits of genetic improvement to the broader aquaculture industry in Pakistan, ensuring widespread adoption and impact.

Step 5: Integrate Superior Strains with Sustainable Aquaculture Practices

To maximize both environmental and economic benefits, future research and development efforts should explore the integration of superior *Labeorohita* strains into sustainable aquaculture practices. This involves promoting environmentally friendly farming systems such as recirculating aquaculture systems (RAS), polyculture, and integrated multi-trophic aquaculture (IMTA).Using genetically improved species in these systems will enhance productivity and minimize environmental damage. The focus on

sustainable practices is essential for long-term resource conservation, reducing the ecological footprint of aquaculture as well as improving food security for increasing populations.

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