Production of intergeneric hybrid of *Catla catla* × *Labeo rohita* and evaluation of genetic and phenotypic variations

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A thesis submitted in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

By

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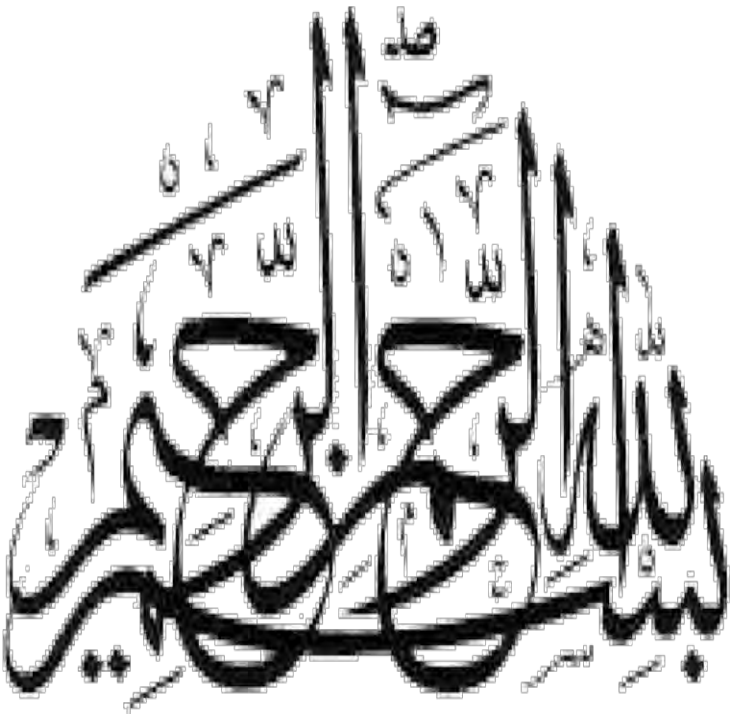

IN THE NAME OF ALLAH, THE MOST MERCIFUL THE MOST BENEFICENT

AND

THE MOST COMPASSIONATE

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This is to certify that the research work presented in this thesis, entitled "Production of Intergeneric Hybrid of *Catlacatlax labeorohita* and Evaluation of Genetics and Phenotypic Variations" was conducted by **Mr. Muhammad Ahmad** under the supervision of **Prof. Dr. Amina Zuberi**. No part of this thesis has been submitted anywhere else for any other degree. This thesis is submitted to the Department of Zoology of Quaid-i-Azam University, Islamabad in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Field of Fisheries and Aquaculture.

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Dedication

I dedicate this humble effort to the

HOLY PROPHET HAZRAT MUHAMMAD

(Peace and Blessing of Allah Be Upon Him)

The reason for the creation of this universe

&

“To my parents who enlightened my darkest hours by the beautiful combination of their encouraging words and invigorating tone”

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List of Abbreviations

Abbreviations	Full Names
°C	Degree celsius
µg/L	Microgram per liter
µl	Microliter
µM	Micromole
ADG	Average daily gain
ANOVA	Analysis of variance
BLAST	Basic local alignment search tool
BSA	Bovine serum albumin
C	*C. catla* (Broodfish)
CC	F1 *C. catla* (Purebred)
CFU	Colony forming unit
CP	Crude protein
CR	F1 hybrid (*C. catla*♀× *L. rohita* ♂)
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphate
DO	Dissolved oxygen
EDTA	Ethylene diamine tetra acetic acid
EFA	Essential fatty acid (s)
FBW	Final body weight
g/dL	Gram per deciliter
GBM	Gain in biomass
HPH	High parent heterosis
hrs	Hours
IBW	Initial body weight
K2p	Kimura 2-parameter
Kg	Kilogram
Kg/ha	Kilogram per hectare

ln	Natural log
LPH	Low parent heterosis
LSD	Least significant difference
m	Meter
M	Molar
mg/L	Milligram per liter
min	Minutes
mm	Millimolar
MPH	Mid parent heterosis
MS222	Tricaine methane sulphonate
MUFA	Monounsaturated fatty acid (s)
OD	Optical density
PBS	Phosphate buffer saline
pH	Potential of hydrogen
PIT	Passive integrated transponder
ppm	Parts per million
PUFA	Polyunsaturated fatty acid (s)
R	*L. rohita* (Broodfish)
RAS	Respiratory burst activity
RC	F1 hybrid (*L. rohita*♀ ×*C. catla*♂)
RPM	Rounds per minute
RR	F1 *L. rohita* (Purebred)
sec	Seconds
SFA	Saturated fatty acid (s)
SGR	Specific growth rate
TDS	Total dissolve solids
TSP	triple super phosphate
Wt	Weight

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Abstract

Hybridization is a simple and well-known approach to genetic improvement via heterosis. However, heterosis in any hybrid comparative to parents is trait-dependent and may not be achieved in every case. Rohu (R) and Catla (C) are the highly culturable freshwater fish species in Pakistan, India, and Bangladesh. They are variable in qualitative and quantitative traits like *C.catla* is fast-growing than *L.rohita* but carcass value, nutritive value, and immunity of *L. rohita* is better than *C. catla*. Here, a comprehensive study was designed to combine the valuable traits of these closely and phylogenetically related species and produce F1 hybrid of superior quality. Induced breeding of these species was conducted and homospecific (R♀×R♂ and C♀× C♂**)** and reciprocal heterologous (*C*♀ × R♂ and R♀ × C♂**)** crosses were made for the production of parent species (RR and CC, rohu and catla respectively) and reciprocal hybrids (RC, *L. rohita*♀, and CR, *C. catla*♀). To evaluate the impact of hybridization, fertilization, and hatching rate, growth performance, blood profile, immunity, disease resistance, thermal tolerance, nutritive value, and morphological traits of reciprocal hybrids (RC &CR) to parental species (RR & CC) were compared. Results indicated statistically comparable and significantly higher fertilization and hatching rate of both purebred cross-types (RR and CC) as compared to hybrid cross-types (CR and RC). Furthermore, both hybrid cross-types also showed statistically similar fertilization and hatching rate. The growth performance data of all cross-types at different developmental stages, both in controlled and semi-intensive culture systems showed the highest final weight, weight gain % and SGR %, of CC followed by CR hybrids while RR showed the lowest values. Moreover, pairwise comparison among hybrids indicated considerably ($p<0.05$) higher weight gain and SGR % of CR hybrid. The relative expression of growth-related genes MyoD, Myogenin, and IGF-1 in all cross-types showed tissue-specific expression, i.e., higher expression of MyoD and Myogenin genes in the muscle compared to IGF-1 that showed higher expression in the liver. The expressions are in accordance with growth performance data, i.e., significantly ($P<0.001$) higher expression of Myogenin, MyoD and IGF-1 genes in the CC (*C. catla*) followed by F1 CR hybrid while the lowest expressions in RR (*L.rohita*). Based on growth performance, all cross-types followed the following rank CC > CR >RC>RR. Both F1 hybrids showed low parent heterosis, with the CR hybrid favorable mid-parent heterosis. Phenotypically, both hybrids (RC and CR hybrids) were intermediate in most of the traits, while some traits resemble their mother species. In morphometric features,

the CR hybrid resembled *C. catla* in body appearance and *L. rohita* in mouth profile. The body of CR hybrid was compressed, relatively deep with a far more convex dorsal profile compared to ventral profile, and like CC, pectoral, ventral, and anal fins were black. However, the body depth of the RC hybrid was wider than RR but not to CC**,** while the head size was smaller compared to CC and somewhat larger than RR. The mouth of both hybrids was terminal with a circular opening. For accurate identification and phylogenetic relationship of all cross-types (RR, CC, CR, and RC), DNA barcoding by mitochondria COX 1 was also conducted. The constructed Neighbour Joining (NJ) tree (K2P) based on COI gene sequence analysis categorized all four crosses into two genetically distant groups. Each hybrid showed an identical phylogenetic relationship with the mother species, i.e., CR hybrids showed the minimum distance with CC**,** and RC hybrid showed the minimum distance with RR. Moreover, the genetic distance between CC and RC hybrid was higher than between RR and RC hybrid**.**

For the evaluation of health status, immunity, and disease resistance against pathogens, a randomly designed experiment under controlled conditions was also designed, and 200 fingerlings of each cross-type after about two weeks of acclimation were evenly distributed in their respective 8 tanks at a stocking density of 2g/L (25 fingerlings/tank). The first two tanks of each cross-type were marked as control without exposure to pathogen while the other six tanks were exposed to the pathogen. Among pathogen exposed tanks, 2 of each were used for evaluation of cumulative mortality (%) during 20 days exposure, the other 2 for the evaluation of immunological indices, and the last two for the temporal and spatial expression of immune-related genes. All fingerlings except in two tanks of each cross-type were exposed to pathogen *Aeromonas hydrophila***,** at a concentration of 2.24×10^7 CFU/g. The control fish were injected with 100µL sterile PBS. For evaluating disease resistance, fish in each group were observed closely for disease symptoms and mortality was recorded. For hematological indices, at random 18 fingerlings of each cross-type were collected from their respective control group while for evaluation of immunological indices, after 24 hrs post-infection, the blood of 18 fingerlings (9/tank), both from control and pathogen exposed groups of each cross-type were collected. Moreover, 6 fingerlings from the last two tanks of each cross-type (3 fish/tank) were captured after 0, 6, 12, 24, 48, and 168 hrs post-challenge, and their liver and muscle were stored in RNA Later™ for gene expression study. Results of this part of the study indicated that RC hybrids had the highest values of RBCs, WBC, MCH, MCHC, HB, and Hct% followed by CR hybrid as compared to parental species. The post-

challenge survival rate showed a similar trend i.e., the highest survival % of the fingerlings of RC followed by CR hybrid while CC fingerlings showed the highest mortality rate. The pre- and post-challenge immunity parameters: serum total protein, IgM, lysozyme level, phagocytic activity, phagocytic index and respiratory burst activity, also showed the highest values in the RC hybrids than CR hybrids while CC showed the lowest values. The spatial and temporal relative expression of immunity-related genes Lysozyme C, Lysozyme G, and TNF-α after challenge to *A. hydrophila* were in accordance to the results of immunity indices and indicated a significantly higher mRNA level of lysozyme C, lysozyme G, and TNF-α genes in the liver and muscle of the fingerlings of RC hybrid followed by CR and RR while fingerlings of CC showed comparatively lower expression. Overall, both hybrids showed higher immunity, and heterosis (LPH, MPH, and HPH) in all studied immunity parameters before and after challenge to *A. hydrophila*. Based on immunity, F1 fingerlings of all cross-types followed the following rank RC > CR >RR>CC before and after pathogen challenge, while based on heterosis, RC hybrid followed the CR hybrid. The critical thermal tolerance (CTmax and CTmin) of all cross-types: RR, CC, and their reciprocal hybrids RC and CR being acclimatized at four acclimation temperatures (22, 26, 30, and 34°C) indicated, comparatively the highest CTmax with the largest total and intrinsic polygon zones as well as the upper and lower acquired thermal tolerance zones of RR followed by RC and CR hybrids. However, CC showed the highest CTmin value and the smallest intrinsic and acquired thermal tolerance zones. Both RC and CR hybrids showed intermediate thermal tolerance with low parent heterosis. The nutritive value of all cross-types indicated a significantly higher percentage of crude protein and fat contents, monosaturated fatty acids (MSFA, 27.45%) and polyunsaturated PUFA (>33%) in the muscle of RR followed by both hybrids, while muscle of CC contained a significantly higher concentration of moisture, ash content, non-essential amino acids (>92%) and the lowest values of MSFA and PUFA. Moreover, both hybrids (RC and CR) showed intermediate values of crude protein and fat contents, MSFA and PUFA with a lower ratio of ω3 to ω6 PUFA as compared to parental species (RR and CC). Additionally, the RC hybrid showed significantly higher concentration of essential amino acids (EAA) especially alanine, yet the CR hybrid showed a significantly higher value of PUFA ($p<0.05$) as compared to both RR and CC. Overall results indicated intermediate growth performance, thermal tolerance, nutritive value, and morphological features while improved immunity and disease resistance of both hybrids compared to both parental species. These results also indicated the higher disease resistance, immunity, thermal

tolerance, and nutritive value of RC hybrid compared to CR hybrids which showed a higher growth rate.

Introduction

Globally, with the increase in population, the demand for food, especially high-quality protein from aquatic sources, is growing considerably. Since the late 1980s, capture fisheries is showing a declining trend due to multiple reasons, including overfishing, pollution, habitat destruction, and climatic changes, etc. Under the current global conditions and environment, further increase in the stock of many major fish species is not be expected, thus to fulfill the demand of the population, the pressure on aquaculture is continuously increasing. It is well established that the biomass of aquatic animals, including fish that can be produced per hectare is far greater than that for land animals. This indicates that aquaculture could play a vital role in global food security (Omole, 2017). Though fish and shellfish production through aquaculture contribute remarkably with global production of 87 Mt (FAO, 2018), even so, still further advancement, i.e., increase in efficiency of aquaculture production is required to meet the food insecurity challenges of the fast-growing world's population, generally in developing countries, like Bangladesh, India, Pakistan, etc.

The increased demand for aquaculture products is continuously increasing the pressure on aquaculture and indicates the need for a more efficient production system. The efficiency of aquaculture production can be improved by acquiring sound management practices and genetic engineering. Substantial improvement has already been made in the management of culture systems, husbandry practices, water quality maintenance, nutrition, disease prevention, diagnostics and treatment, as well as up to some extent, in the genetic improvement of aquaculture species.

For many years, the genetic intervention has been used to improve animal and agricultural plant production worldwide (Dunham et al., 2000; Nguyen et al., 2016). From the last three decades, genetic tools are gaining importance in aquaculture to overcome many different production challenges. Aquaculture genetics demonstrates the immense potential for improving production efficiency, increasing production in a way that meets goals set for sustainable development of aquaculture for the new millennium. Fish genetic programs were first initiated in the 1900s, after the development of the basic principles of genetics and quantitative genetics. Yet, due to small-scale aquaculture and insufficient knowledge of fish genetics, genetic enhancement programs did not practically start until the 1960s. From 1960 to 1980, many developments have been made in the field of fish genetics like selective

breeding of common carp in Israel (Wohlfarth, 1986), Channel catfish in the USA (1970-1980), various salmonids (TrgveGjedrem, Harold Kincaid and, later, William Hershberger, 1970s) in different countries as well as, development of sex-reversal technology for tilapia (Guerrero III and Shelton,1970s). Since the 1980s, with the involvement of molecular-based knowledge and biotechnology, the intervention of genetics in aquaculture has gained momentum and started to show the impact on production traits of culturable species (Dunham et al., 2000).

Now it is well established that by genetic research and application of genetic enhancement programs, many constraints of aquaculture sustainability including resource utilization, slow growth and high mortality of fish from diseases, pollution and temperature fluctuation, inefficient feed conversion, low fecundity, poor reproduction, inability to tolerate low DO level, inefficient harvest or post-harvest losses can be addressed (Dunham, 2011). Nowadays, the development of new high-value and fast-growing breed of aquatic organisms, which can flourish in diverse environmental conditions and contribute to boosting up production, is considered as one of the most required processes for promoting aquaculture and meeting the future food challenges of the growing population.

Production of genetically improved fish is a prerequisite for advancing aquaculture (Shah 2004; Dunham, 2011; Gjedrem and Morten, 2018) and it can be achieved through various methods including, strain evaluation and selection, inbreeding, selective breeding, and hybridization (Xu et al., 2015). Through these techniques, not only the aquaculture production can be enhanced but the marketability of culturable species, culture environment, and the conservation of natural resources could also be improved (Moses et al., 2005). For the initiation of a genetic enhancement program and rapid initial genetic improvement, the first step is the selection and use of best-performing domestic strains (Dunham, 2011). A strain is a population of fish within a single species that has the same history, the same origin, and has at least one trait or a suite of traits that make it unique or make it different from other strains. It is well documented that different strains of fish display large amounts of variability for many different traits like body conformation, growth rate, color, age of maturity, disease resistance, dressing percentage, fecundity, time of spawning, (Smitherman and Dunham, 1985; Coe et al., 2008). For instance, infectious pancreatic necrosis (IPN) virus resistance variability has been reported in different strains of rainbow trout (*Oncorhynchus mykiss).*

(Okamoto et al., 1993). Similarly, variation in caudal fin disassociation, swimming equilibrium, higher temperature of Barramundi, (*Lates calcarifer)* strains (Newton *et al*., 2010), and in upper and lower thermal tolerance limit, (*L. rohita*) strains (Syed et al., 2018) are well documented. Strain evaluation is imperative, since strain affects other genetic improvement approaches, like hybridization, sex control, and genetic engineering.

Hybridization is another well-known approach of genetic improvement via heterosis (Bartley et al., 2001; Scribner et al., 2001; Rahman et al., 2000, 2005, 2013; Facon, et al., 2005; Ågren et al., 2018; Zhong et al., 2019). It is one of the low-cost and potential tools of improving genetic traits of fish, involving combining the desirable traits of two different organisms (strains) or selected species and produce a new generation of totally different and superior quality organisms known as hybrids (Ayinla and Nwadukwe, 2003), guarantees to survive in competition with parents (Mallet, 2007). Generally, hybridization leads to genomic changes like chromosomal rearrangement, gene silencing, and differential gene expression (Josefsson et al., 2006; Tirosh et al., 2006; Otto, 2007; Hoffmann and Sgrò, 2011; Morales and Dujon, 2012). Generally, the F1 or first-generation hybrids own a genetic combination of both parents, which may lead to different ontogenetic trajectories and produce phenotypic variation (Corse et al., 2012). The prime goal of hybridization in animal husbandry is to acquire the favored or selected traits of each parent in a hybrid. Hybrid vigor or positive heterosis is achieved, when a hybrid possesses characteristics superior to both parents (Rahman et al., 2013) Heterosis or hybrid vigor could arise due to intra-allelic interactions, inter-allelic interactions, or superior alleles combination at multiple loci (Hochholdinger and Hoecker, 2007). The intensity of heterosis is generally higher when parental species are genetically distant from each other or are highly inbred (Shikano et al., 2000; Wang and Xia, 2002; Hochholdinger and Hoecker, 2007). Additionally, the selection of the strain used as a sire or dam in the cross may also affect the expression of heterosis (Bentsen et al., 1998). Therefore, specific capacities in hybrids could be improved by using better strains as a dam or sire (Perry et al., 2004; Wang et al., 2006).

Hybridization is an effective approach to deal with a variety degeneration (decline) and produce a superior variety (Wang et al., 2019). It could be used to improve performance via heterosis, like faster growth rate (McEntire et al., 2015), improved feed utilization (Daudpota et al., 2016) as well as increased immunity and improved disease resistance

(Simkova et al., 2015). The crossbreeding of different sub-species within species or different strains of a species (individuals of the same species with different characteristics) is called intra-specific hybridization. The phenotypic and genotypic variations in hybrids are the results of the combination of the genome from different sub-species (Wang et al., 2019). It is a common technique for producing genetically improved livestock animals (Mohammed et al., 2005; Musa et al., 2006; Saadey et al., 2008; Bekele et al., 2010; Farahvash et al., 2011; Razuki and Al-Shaheen, 2011). Although it could increase the growth rate, however heterosis may not be achieved in every case. For instance, a 22 % and 55% increase in growth rate was observed in the crossbreeds of rainbow trout and channel catfish respectively, (Dunham and Smitherman, 1983; Dunham, 1996). The cross-breeding of *Clarias anguillaris* also showed better performance of the progeny (Onyia et al., 2010). Similarly, higher adaptability to warmer conditions was noted in the cross-breed of European catfish (*Silurus glanis*) (Bartley et al., 2001), while, cross-breed of walking catfish, (*Clarias macrocephalus*), showed improved resistance to *A. hydrophila* infections (Srisapoome et al., 2019). However, cross-breeds of Chum salmon (Dunham, 1996) and Atlantic salmon (Friars et al., 1979; Gjerde and Refstie, 1984) did not show a significant heterosis effect for either growth or survival rate.

The mating or crossing of two distant or different species usually from within the same genus or higher-ranking taxa is called Inter-specific or distant hybridization. The genotypic and phenotypic variations in hybrids are the results of the combination of the genome of different species (Wang et al., 2019). It seems interspecific/distant hybridization has greater potential to produce variation in the genotype and phenotype of hybrid in contrast to intraspecific hybridization. It has long been practiced in aquaculture to combine desirable traits from two species, to transfer valuable characteristics from one species to another for improving production by increasing growth rate, improving flesh quality, manipulating sex ratios, producing sterile animals, increasing disease resistance and environmental tolerance, improving food conversion rate and improving a variety of other traits by taking the advantages of sexual dimorphism, thus to make aquaculture more profitable (Bartley et al., 2001, 2004; David and Pandian, 2006). Generally, the offspring exhibit characteristics and traits of both parents and often sterile, thus preventing gene flow among the species.

Interspecific hybridization is an approach that facilitates adaptation, evolutionary novelty, and the origin of new species (Rieseberg and Willis, 2007; Mallet, 2008; Abbott et

al., 2013). Over the last three decades, many examples of animal hybridization have been reported in nature (e.g. invertebrates; Howard, 1986: Heath et al., 1998; Yao et al., 2015; Zhang et al., 2017; birds, Grant and Grant, 1992; Burrell et al., 2016; David et al., 2018; Ottenburghs, 2019; fish, Avise and Saunders, 1984; Hatfield and Schluter, 1999; Taylor et al., 2002; Deines et al., 2014; Khaefi et al., 2018; Vasil'eva and Vasil 2019). Generally, interspecific hybridization is more common in fish as compared to other vertebrates (Allendorf and Waples, 1996; Scribner et al., 2001). There are about 32,500 fish species, representing the largest vertebrate group (Cossins and crawford, 2005). This large number represents the important genetic resource that provides a variety of alternative parents for interspecific hybridization and production of improved variety. Although reproductive isolation between species exists in nature which maintains the balance and stability of species, however various factors like the unequal abundance of the two parental species, external fertilization, weak behavioral isolating mechanisms, competition for narrow spawning ground, and decreasing habitat complexity appear as driving forces in the higher occurrence of natural hybridization among closely related fish species (Hubbs, 1955; Campton, 1987; Wang et al., 2019).

Natural hybridization is an important evolutionary or ecological process, that contributes to speciation and has been reported in several taxa, especially in fish (Scribner et al., 2000; Abbott et al., 2013) and plants (King et al., 2010). For instance, the presence of several interspecific hybrids like a hybrid of Nile tilapia (female) × blue tilapia (male) (Deines et al., 2014), *Pundamilia pundamilia* × *P. nyererei* (Taylor et al., 2002) in natural water bodies (lakes and rivers) indicate the role of interspecific hybridization in speciation. Generally, hybridization among species is linked with human intervention or the destruction of habitats of parental species. Natural hybridization events are normally occurring during the spawning event without human interference within the species' native range, while human-induced hybridization event occurred either by directed manipulations of the environment (Construction of dams and canals, pollution, etc.) that resulted in the loss or alteration of spawning grounds, determined translocations of species from their historic native range or purposeful hybridization, i.e., artificial crosses between species for desirable traits (Scribner et al., 2001). Among anthropogenic factors, aquaculture is the most prevalent one that contributes about 39% of interspecific hybridization (Scribner et al., 2001)

The directed inter-specific hybridization in aquaculture is common among species in three families Ictaluridae, Cichlidae, and Cyprinidae. During the 1990s, there are about eighteen cyprinid species that are used to form nineteen hybrid combinations (Hulata, 1995; Lutz, 1997). By 2017, the Chinese government approved 83 new genetically improved fish varieties including 40 hybrids. This shows the importance of hybridization technology in the production of improved aquaculture products. In aquaculture, the human-directed inter-specific hybridization to increase/improve growth rate, disease resistance, environmental tolerance, flesh quality, and palatability, as well as the production of sterile animals, has long been practiced to make the aquaculture industry more profitable (Khajarern and Khajarern, 1997; Rahman et al., 1995; Bartley et al., 2001 & 2004; David and Pandian 2006; Rehman et al., 2013; Al Mamun and Mahmud, 2014; Wang et al., 2019). Earlier, the majority of the research work on hybridization was performed for salmon fish, however, these species did not show hybrid vigor or commercial importance. Therefore, hybrids in these fish do not attract fish culturists (Bartley et al., 2000; Bryden et al., 2004; Miller et al., 2004).

In the last decades, with the expansion and intensification of fish production throughout the world, interspecific hybrids also gained importance and now playing a significant role in global production through aquaculture. About 35 interspecific hybrid fish from 15 families have been produced so far. Some of which are popular in aquaculture e.g., hybrid striped bass is commonly known as whiterock bass (*Morone saxatilis* × *M. chrysops)* in the USA, hybrid characids in Venezuela, hybrid catfish (*C. gariepinus* x *C. macrocephalus*) in Thailand, hybrid tilapia (*Oreochromis niloticus* × *O. aureus*) in Israel, and Tambacu hybrid (*Colossoma macropomum* x *Piaractus mesopotamicus)* in Brazil while others are in the process of evaluation (Bartley et al., 2000, 2001 & 2004; Diogo et al., 2012; Rahman et al., 2013). The better knowledge of reproductive biology of fish, improvement in artificial breeding and *in vitro* fertilization techniques are the main factors which are encouraging the aquaculturists to use interspecific hybridization technique and produce hybrids with improved quality traits over their pure parental species (Rahman et al., 2018)

In aquaculture, human-directed inter-specific hybridization has been practiced to produce hybrids of superior traits resulting from heterosis or to mix targeted traits of both parental species (Falconer and Mackay, 1996; Rahman et al., 2018). Heterosis or hybrid vigor has a tremendous economic value in the aquaculture and agriculture sectors. It manifested in

various ways like increase in survival rate, growth rate, fertility rate or reproductive ability, disease-resistance, thermal tolerance, etc. Generally, interspecific hybridization via heterosis (hybrid vigor) may also have positive effects on fitness at the individual level and/or a higher degree of genetic variance for particular phenotypic traits at the population level (Facon et al., 2008; Benvenuto et al., 2012). These features can contribute to the adaptation of hybrids to heterogenous or novel environments better than the parental species (Benvenuto et al., 2012)

The difference in the performance of the hybrid relative to the average of the parent species is termed as the mid-parent (MP) value, while the deviation of the hybrid relative to MP is the relevant value. Practically, high-parent heterosis (HPH), which quantifies the superiority of the hybrid relative to the best parent, is the important target in the genetic enhancement program. Heterosis in contrast to inbreeding depression generally increases the heterozygosity and its measures are phenotype-dependent. For instance, some interspecific hybrids of fish may display increased growth rate, size, immunity, and other desirable fitness traits, but are often sterile, thus have reduced fecundity. According to Flint-Garcia et al. (2009) heterosis in any hybrid relative to its parents is trait-dependent and that hybrids could not be simply classified as heterotic or non-heterotic.

Heterosis can be explained by dominance, overdominance, or epistasis. The dominance hypothesis first postulated by Davenport (1908), explains the hybrid vigor due to cancelling /masking of inferior or deleterious recessive alleles of one parent by beneficial or superior dominant alleles of others. Nevertheless, the overdominance hypothesis postulates the superiority of hybrid due to diverse intra-allelic interaction in contrast to when alleles occur in the homozygous state. Though various molecular mechanisms have been reported for such interactions, however, none is obvious (Kato et al., 2006). If overdominance is the major source of heterosis, inbreeding strategies where the major objective is to maximize heterozygosity, then the result will be the best performance. However, if dominance or epistasis is the main mechanism of heterosis in the natural or human-directed breeding populations then individuals will perform equally to any hybrid because of the fixation of favorable alleles (Reif et al., 2007). For instance, due to over-dominance male F1 hybrid (Nile tilapias ♀ × blue tilapias ♂) compared to both parents showed higher expression of growth hormone (an indicator of growth) while in female F1 hybrid, maternal dominance

contributed to the higher GH expression as compared to blue tilapia and statistically similar to Nile tilapia (Zhong et al., 2019).

The results of interspecific hybridization could be variable and depend on the genetic makeup, gamete compatibility, crossing patterns, and gene flow patterns of the parental species (Rahman et al., 2013). According to many scientists, the phenotypes of hybrids are affected by several changes like transposon activities, loss of a fragment of the DNA, and variation in the levels of DNA methylation (Kidwell and Lisch, 1998; Han et al., 2003; Abid et al., 2011). Generally, heterosis, makes the hybrid speciation successful and associated with gene expression patterns. For instance, the unique phenotypes like fast growth rate (Sun et al., 2016; Zhong et al., 2019) and strong resistance of fish to pathogens (Zhang et al., 2006) are related to the overall expression of homologs in hybrids (Zhang et al., 2006 & 2019). In hybrids, the gene expression could be additive and nonadditive (Ren et al., 2016; Zhu et al., 2017b). The additive gene expression indicates the reduced differences and hybrids appear close to the average of their parents (Bell and Travis, 2005). The nonadditive gene expression has been regarded as a specific expression pattern for heterosis (Li et al., 2015). The non-additive gene expression in hybrid could be confirmed, when the expression is higher or lower than the mid-parent value. Besides, overall expression pattern, allele-specific expression (ASE) i.e., sequence variation in regulatory regions is another mechanism of heterosis (Shao et al., 2019) and reported in hybrids (Cheng et al., 2016; Zhong et al., 2019).

Indeed, not all of the hybrids are contributing to commercial aquaculture production. Several are produced intentionally or unintentionally for release in open water bodies like floodplains under the government restocking programs or to increase sport fishing (Hussain and Mazid, 2001). For instance, hybrids produced for angling purpose include white bass × striped bass (*Morone chrysops* × *M. saxatilis*), muskellunge × northern pike (*Esox masquinongy*× *E. lucius*), green sunfish × bluegill sunfish (*Lepomis cyanellus* × *L. macrochirus*), largemouth bass × smallmouth bass (*Micropterus salmoides* × *M. dolomieu*), walleye × sauger (*Stizostedion vitreum* × *S. canadense*), channel catfish and blue catfish (*Ictalurus punctatus* × *I. furcatus*), brook trout × lake trout (*Salvelinus*× *fontinalus* and *S. namaycush*), green sunfish × redear sunfish (*L. cyanellus* × *L. microlophus*), brown trout × rainbow trout (*Salmo trutta* × *Oncorhynchus mykiss*). However,

in several cases, these hybrids decrease the native parental species and hampered conservation efforts (Scribner et al., 2001).

Although human-directed interspecific hybridization is a potential genetic tool in aquaculture to produce fish with desirable phenotypes from two different species, however, the success and results of hybridization vary considerably, i.e., from non-viable embryonic production to viable and sterile to fertile progenies (Rahman et al., 2013; Wang et al., 2019). For instance, the success of interspecific hybridization depends on the phylogenetic karyotypic relationships (Aluko, 1998). The incompatibility of chromosomes of parent species is the major constraint to any successful hybridization (Adah et al., 2014). Like results of hybridization, heterosis is also uncertain, depending on various factors including strains used as sire or dam (Zhong et al., 2019). Thus it is not guaranteed to be successful in improving the performance of offspring. For example, the performance of F1 hybrids of salmonids (Bartley et al., 2000; Bryden et al., 2004; Miller et al., 2004), char (Dumas et al., 1992 & 1996; Cyr et al., 2018) are often less as compared to parental species, while a hybrid of tilapia (*O. niloticus*× *O. aureus)* showed faster growth rate as compared to parental species when *O. niloticus* was used as a dam. However, no such growth vigor could be found in the reciprocal hybrid (Pruginin et al., 1975).

Many scholars have carried out many studies on interspecific hybridization and reported the impact of hybridization on different traits of fish. Most studies conducted their experiments on the families of Salmonidae Cyprinidae, Poeciliidae, and Centrarchidae (Schwartz, 1981) and used distant species having a similar number of chromosomes. For instance, F1 hybrids resulting from the crossing of *O. niloticus* ♀ × *O. aureus* ♂ (♀ and ♂ chromosome number, 2n=44) were all male and showed fast growth rates, high yields, and strong disease resistance (Xu, 1984; Wang et al., 1989). Similarly, F1 hybrids derived from the crossing of *I. furcatus*♀ × *I. punctatus* ♂ (2n of both ♀ and ♂ =58) showed obvious heterosis and 30% faster growth rate as compared to parental species (Dunham and Argue, 1998), while F1 hybrids obtained from reciprocal crossings of *M. chrysops* × *M. saxatilis* (2n of both ♀ and ♂ =48) showed stronger stress and anti-disease resistance and grew more quickly than parental species (Gaylord and Gatlin III, 2000). Moreover, successful F1 hybrids derived from the crossing of *O. niloticus* × *O. mossambicus* (2n of both ♀ and ♂ =44) (Tang et al., 2006), scattered mirror carp♀ × red crucian carp ♂ (2n of both parents=100) (Zhou et

al., 2008), *Erythro culterilishaeformis* ♀×*Ancherythroculter nigrocauda* ♂ (2n of both parents =48,) (Li, 2013), *Siniperca scherzeri* ♀ ×*Siniperca chuatsi* ♂ (2n of both parents=48,) (Qian et al., 2016), and *Epinephelus fuscoguttatus* ♀× *E. lanceolatus* (2n of both parents =48) (Zhang et al., 2018) also had an equal number of chromosomes like their parents. These results are consistent with the breeding rules proposed by Wang et al. (2019) in the one-step breeding technology.

Generally, it is considered that interspecific hybridization produces infertile lineages because of the presence of reproductive isolation in the crossing of distant species. However, this is not true in all cases. Based on many studies (Liu, 2010; Qin et al., 2010; He et al., 2012; Hu et al., 2012; Song et al., 2012; He et al., 2013; Qin et al., 2014; Xiao et al., 2014; Wang et al., 2015; Xu et al., 2015; Chen et al.,2016; Liu et al., 2007; Wang et al., 2017; Hu et al., 2018; Wang et al., 2018), Wan et al. (2019) suggested the genetic rule of interspecific hybridization. According to them, the results of hybridization depend on the numbers of maternal and paternal chromosomes. Generally, there are two types of patterns in hybridization, one in which both parents have the same number of chromosomes while in other, parents involved in hybridization have different number of chromosomes. When the paternal chromosome number is less than maternal chromosomes, then the F1 hybrid can overcome the reproductive barrier and established autodiploid and autotetraploid lineages. If the number of chromosomes of both parents (maternal and paternal chromosomes) are equal then, the F1 hybrid breakthrough the reproductive barrier and established allodiploid and allotetraploid lineages. However, when the number of maternal chromosomes is less as compared to paternal chromosomes, then progeny of hybridization have little chance to survive (Liu, 2010 & 2014; Song et al., 2012).

By the application of genetic rules, a fish breeder can avoid the unexpected results of hybridization, (no or fewer progeny) and predict the relationship between parent and offspring (Wang et al., 2019).

The genetic rules indicated that at the chromosomal level, matching of the chromosome numbers of the parents is involved. The degree or strength of matching the number of chromosome of the parents influences the nucleus-cytoplasm and nucleus-nucleus compatibilities of F1 hybrid which in turn affects the survival rate of the F1 hybrid. These nucleus-nucleus and nucleus-cytoplasm compatibilities reflect the similarity between the

maternal and paternal genomes as well as the maternal genome-cytoplasm and the paternal genome-cytoplasm (Wang et al., 2019).

According to the genetic rule reported by Wang et al. (2019), in Intraspecific hybridization, the number of maternal and paternal chromosomes is equal and compatibilities between the maternal and paternal genomes are fairly good, therefore the survival rate of F1 hybrid is generally high. However, in interspecific hybridization, when the number of maternal and paternal chromosomes is equal, then in F1 hybrid, the compatibilities between the maternal and paternal genome as well as, the maternal genome-cytoplasm with the paternal genome-cytoplasm are good, therefore F1 hybrid shows a higher survival rate. Furthermore, when the maternal chromosome number is higher than the paternal chromosomes, then the maternal genome is in the dominant position. In this case, the compatibilities of the maternal and paternal genome as well as, the maternal genome-cytoplasm with the paternal genome-cytoplasm will be deceased. Therefore, the F1 hybrid of this cross will show a certain survival rate. However, when the maternal chromosomes are less in number as compared to paternal chromosomes, then the paternal genome is in the dominant position. In this case, the compatibilities of the maternal and paternal genome as well as, the maternal genome-cytoplasm with the paternal genome-cytoplasm will decrease further. Thus F1 hybrid of this cross will show a poor survival rate.

Many interspecific hybrids have been produced, however, most of them are of no applied/commercial importance (Bartley et al., 2000; Rahman et al., 2018; Wang et al., 2019). However, the number of hybrids showed desirable characteristics from both parents and appeared suitable for aquaculture (Issa et al., 1986; Kowtal, 1987; Salami et al., 1993; Basaravaju et al., 1995; Harrell, 1998; Rahman et al., 2013). Among them some showed intermediate growth e.g., Channel Catfish × Blue Catfish Hybrids (Neely et al., 2020), sturgeon × sterlet (Shivaramu et al., 2019). *C. gariepinus* × *C. anguillaris* (Akinwande 2011), Nile tilapia (*O. niloticus*) × Blue tilapia, (*O. aureus*) (SamyYehya and El-Zaeem 2011), *Heteropneustes fossilis*♀ × *C. batrachus*♂ (Jothilakshmanan and Marx 2013), *C. catla* ×*Labeo fimbriatus* (Basavaraju et al 1995), *C. garìepìnus*×*H. longifilis* (Valenciennes, 1840 and Burchell, 1822), *H. longifilis* x *C. gariepinus* (Legendre et al., 1992), *C. carpio* × *C. catla, L. rohita*× *C. mrigala, L. rohita*× *C. carpio* (John and Reddy, 1986; Khan et al., 1989). *C. catla*♀ × *L. rohita*♂ (CR) (Alikunhi et al., 1971; Varghese and Sukumaran 1971;

Chaudhuri, 1971; Natarajan et al., 1976; Reddy and Varghese 1980; Reddy 2000; Sarder et al., 2014; Al-Mamun and Mahmud, 2014) and *M. chrysops* × *M. saxatilis* (Smith, 1988). while other superior to parental species, for example, sterlet (*Acipenser ruthenus*) × Siberian sturgeon (*A. baerii*) (Shivaramu et al., 2019), *Epinephelus fuscoguttatus* × *E. lanceolatus* (Zhang et al., 2018), *M. chrysops*♀ × *M. saxatilis*♂; *Haliotis discus hannai* ♀ × *H. discus discus* ♂ (Hoshikawa et al., 1998), *E. fuscoguttatus* × *E. polyphekadion* (James et al., 1999), CyCa nucleocytplasmic hybrid × scattered mirror carp (Liu et al., 2017) grow better than the male and female parents. However, some show less growth than both parents for example *C. gariepinus*× *H. longifilis* (Ataguba et al., 2010) in plastic aquaria and salmonids has shown that first-generation hybrids often perform less well than parental lines (Bartley et al., 2000; Bryden et al., 2004; Miller et al., 2004),

In 2001, Scribner et al. collected the distant hybridization in fish data and reported that species within the family Cyprinidae were hybridized most frequently and accounted for about 40% of all hybrids in contrast to Centrarchid (20%), Salmonidae (8%), Percidae, Catostomidae, and Poeciliidae (4%), Cichlidae, Clariidae, and Esocidae (3%), Cyprinidontidae (2%), and all others (1%). They reported that 68 Cyprinid species were involved in 56 inter-species hybrids. According to Purdom (1993), within the family Cyprinidae, natural hybridization is a quite common phenomenon. For instance, in Australian and Canadian waters, natural hybrids of two exotic species common carp × gold were most frequently observed (Taylor and Mahon, 1977; Hume et al., 1983). Similarly, natural hybrids of *L. rohita* × *C. catla, C. catla* × *L. rohita, L. calbasu* × *C. catla, L. fimbriatus* × *L. gonius* have also been reported by many scientists in the water bodies of India and Pakistan (Desai and Rao, 1970; Prasad, 1976; Natarajan et al., 1976). Generally, cyprinids species spawn in groups and their spawning activities show a mutualistic or commensal relationship with other species often within very restricted areas or spawning substrates (Rahman et al., 1995; Khajarern and Khajarern, 1997). Therefore higher occurrence of natural hybrids could be due to the scarcity of one species and dominance of a related species nearby (Hubbs, 1955) or overcrowding of species in the spawning ground. In restricted or limited spaces (may be due to the construction of dams across rivers), there is a greater chance of fertilization of ova of one species with sperm of other species (Nagpure et al., 2001). Indian major carps have a more compatible genomic structure, therefore natural hybridization more likely occurs in these species (Zhang and Reddy, 1991)

Species within the cyprinids have been more frequently hybridized purposely in many countries including China, Bangladesh, India, and Burma, to improve the growth rate, production per acre, palatability, disease resistance, and economic value of the product. From the last several decades, many investigators especially from China and India are extensively involved in conducting artificial hybridization among cyprinids of different genera and even families and concluded that inter-families hybridization is not much successful (Wu, 1990; Wang et al., 2018). Although, some hybrids between subfamilies showed survival up to hatching, beyond which higher rate of abnormalities appeared and survived fish were mostly sterile (Wu, 1990; Wang et al., 2018).

Generally, intergenic hybrids, produced by the mating of two species having a similar number of chromosomes and karyotypes, like, *Cyprinus carpio* × *Carassius auratus* and *Hypophthalmichthys molitrix* × *Aristichthys nobilis*) are viable and even fertile or in some cases only males showed sterility e.g. *C. carpio* × *C. carassius* (Makino et al., 1958). However, when species had different number of chromosomes or the karyotypes, then interspecific hybridization failed, eg., *C. carpio* × *Ctenopharyngodon idella* (Wu, 1990). From 1980 to 1995, extensive work on interspecific hybridization was carried out in China and several hybrids with valuable traits for aquaculture like accelerated growth of *H. molitrix* × *C. idella* and *C. carassius* × *C. carpio* hybrids (Liu et al., 1986) and low-temperature tolerance of *Cirrhinus molitorella* × *Sinilubeo decorus* hybrid (Wang et al., 1984) had been selected and being introduced in the culture system of China (Wu, 1993).

During the 1970s, a successful crossbreeding program among five cyprinids; *C. carpio*, *H. molitrix*, *Aristichthys nobilis*, *C. idella*, and tench) was carried out in Hungary to produce hybrids suitable for the natural waters (Bakos et al., 1978). Although all interspecific hybrids among Chinese carp themselves and with *C. carpio* were developed, but, showed variation in their viability. For example, hybrids of *C. carpio* × *H. molitrix* and *C. carpio* × *C. idella* and *C. carpio* × trench were viable as compared to their reciprocal hybrids. However, all interspecific hybrids produced from reciprocal crosses of *C. idella* × *A. nobilis* and *H. molitrix* × *A. nobilis* were viable. Whilst hybrid and reciprocal hybrid from reciprocal crosses of *H. nobilis* × *C. idella* showed gross malformation and lower viability (Hulata, 1995). It indicated that among Chinese carps, crosses between *C. idella* and *A. nobilis* (Marian and

Krasznai, 1978) as well as between *H. molitrix* and *A. nobilis* (Issa et al., 1986) showed the most promising results and appeared as most suitable for aquaculture.

The progeny from reciprocal crosses of bighead carp and silver carp showed a higher survival rate and yield as compared to parent species. Moreover, hybrids were fertile and farmers showed preference to culture hybrids in contrast to silver carps. However, due to widely spaced gill rakers, these hybrids showed lower efficiency in controlling microalgal blooms (Panjusova and Tselikova, 1983; Spataru et al., 1983). In contrast to these hybrids, hybrids from reciprocal crosses of bighead carp and grass carp contain both diploids and triploids hybrids. The diploid hybrid showed reduced viability (Marian and Krasznai, 1979; Beck et al., 1980; Sutton et al., 1981; Allan and Stanely, 1983), while the triploid hybrid was attracted by the U.S. biologists, who were interested in grass carp for controlling aquatic vegetation without compromising its naturalization in natural ecosystems (Dunham, 2011).

In the 1960s hybridization between the crucian carp (*Carassius carassius*) and other cyprinid species has also been explored in Japan and China. It is documented that hybrids between *C. carpio* ♀ and *C. carassius* ♂ were viable (Kafuku, 1968; Kafuku and Matsushima, 1968) and the reciprocal hybrid (*C. carassius* ♀× *C. carpio* ♂**)** attracted the fish farmers because of faster growth rate (Wu, 1993). According to Rothbard (1993), although *C. carpio* ♀ × *C. Carassius* ♂ showed limited potential for food fish production, however, hybrids were produced for use as baitfish. Experiments on the hybridization of Indian major carps (*L. rohita*, *C. catla*, and *C. mrigala*), *C. carpio*, and Chinese carps (*C. carpio*, *H. molitrix*, *C. idella*, and *H. nobilis*) were initiated in 1958 (Chaudhuri, 1961). The interspecific and intergeneric hybridization among Indian major carps themselves, with Chinese carp and with *C. carpio* indicated that generally, the interspecific hybrids of *L. rohita* with Chinese carps (silver carp, bighead carp, and grass carp) were short-lived and did not show any genetic advantage over the parent species (Kowtal, 1987; Jena, 2006). Moreover, hybrids of *C. carpio* ♂ × *L. rohia* ♀ and *C. carpio* ♂ × *C. mrigla* ♀ although, both crosses showed good fertilization rate during incubation, their development ceased, and most of the embryos died. However, the reciprocal hybrid in which *C. carpio* ♀ cross showed higher fertilization and hatching rate, and hybrids were sterile and showed more maternal traits (Khan et al., 1990).

The hybridization experiments further indicated the higher compatibility of Indian major carps for hybridization. The literature revealed the artificial production of about six

interspecific hybrids and 13 intergeneric hybrids among the four species of Indian major carps belonging to the three genera (*Labeo*, *Catla*, and *Cirrhinus*) (Reddy, 1999; Froese and Pauly, 2018a). Among these, at least six intergeneric hybrids were with *L. rohita* (Khan and Jhingran 1975; Tripathi 1992). Although, several crosses like *L. rohita* × *C. catla*, *L. rohita* × *C. mrigala*, *C. catla* × *C. mrigala*, *C. catla* × *L. calbasu*, *C. catla* × *L. fimbriatus*, *L. fimbriatus* × *C. catla*, *L. calbasu* × *C. mrigala* showed successful fertilization and some promising traits from the aquaculture point of view. However in terms of production trait i.e., growth rate out of 13 intergeneric hybrid only 4 hybrids (*L. rohita* × *C.catla*, *C. mrigala* × *C. catla*, *L. rohita* × *C. mrigala*, *L. fimbriatus* × *C. catla*) possessed this useful trait (Ibrahim, 1977; Basavaraju and Varghese, 1980)

Among cyprinids, Indian major carps e.g. rohu (*L. rohita*), thaila (*C. catla)*, and Mrigal (C. *mrigala)* are the most important, prestigious, commercial fish species in India, Pakistan, and Bangladesh. These species have a prime position in aquaculture due to their growth rate, maximum market demand, taste, and consumer acceptability as food (Pallipuram, 2020). At present, these species (*L. rohita*, *C. catla*, and *C. mrigala)* forms an integral component in three, four, five, and six species polyculture system and culture in combination with grass carp (*C. idella)*, silver carp (*H. molitrix*), bighead carp (*A. nobilis*) and common carp (*C. carpio*) (Rahman, 2008a), In 2001, the contribution of major carps in total freshwater fish production was 67% (ICLARM, 2001) with about 3.02 million tonnes of total production (FAO, 2006). Indian major carps, though originally inhabitants of the Ganges River but also widely distributed in the rivers of India, Pakistan, Bangladesh, Nepal, and Burma. In India, carp culture alone contributes about 87% of the total freshwater production (Bias, 2018).

Among the carp species, *L. rohita* (commonly known as rohu in Pakistan) is widely distributed throughout Pakistan and also found in India, Bangladesh, Bhutan, Sri Lanka, Nepal, Malaysia, Afghanistan, Maldives Japan, China, Philippines (FAO, 2006). It is a eurythermal species that does not thrive at temperatures below 14 °C (FAO, 2009). It is a fast-growing species and attains about 35-45 cm total length and 700-800 g in one year under normal culture conditions. Generally, in polyculture, its growth rate is higher than that of mrigal but lower than catla (Sarder et al., 2014). It is an important aquaculture freshwater species in South Asia (FAO, 2018) and is also prized as a game fish (Dahanukar, 2011).

Hundreds of years ago, traditionally, it was cultured in small ponds, and nowadays commercially it is cultured in combination with other major and /or Chinese carps, especially, *C. catla* and *C. mrigala*, *C. carpio*, and exotic species such as *O. niloticus*, *H. molitrix*, *C. idella* in earthen ponds and reservoirs (Rahman, 2008; Ali et al., 2017). It has been introduced beyond its native range in ponds and reservoirs for aquaculture production. Because of the high nutritional value, taste, and fast growth, it was introduced intentionally in other countries like Philippines, Japan, and the former USSR (Jhingran, 1982; Froese and Pauly, 2018). Generally, in polyculture, the growth rate of *L. rohita* is lower than *C. catla* but higher than mrigala. Pakistan and India introduced this species into almost all river systems, and now it also occupies a vital position in the polyculture system. Commercially, it is an ideal planktivorous component in the polyculture fish farming system and only in India, it contributes more than 60% of total carp production (Mohanta et al., 2008)

Globally, rohu contributes about 4% of total aquaculture production of major species and 15% to the world's freshwater aquaculture production (FAO, 2009; FAO, 2018). Among the higher producer country of carps, India is the largest producer of the rohu, then Bangladesh, while Myanmar and Thailand produce to a lesser extent (FAO, 2006). The contribution of rohu in 2001 among the total Indian major carps production was about 35% (FAO, 2001). The compatibility of rohu with other carps, like *C. catla* and *C. mrigala* made it the best candidate for freshwater carp in polyculture systems. When cultured, it does not breed in the lake ecosystem, so induced spawning is necessary (Achaya, 2003).

Rohu has a broader feeding niche, which extends from column to bottom, therefore it is usually stocked at relatively higher levels than the other two species. Rohu takes food from the middle layer of habitat (Talwar and Jhingran, 1991). During the fry stage, rohu predominantly feeds on zooplankton, while showing negative selection for phytoplankton. However, fingerlings and adults have a strong positive selection for phytoplankton and show negative adaption for zooplankton (Ahmed et al., 2007). Furthermore, the food selection and feeding relationship of rohu in polyculture system, i.e., along with *C. mrigala* and *C. catla* indicated that in the adult stage it prefers to eat both plankton and periphyton (Saikia et al., 2013).

The body of *L. rohita* is moderately elongated and bilaterally symmetrical. Its dorsal profile is more arched than the ventral profile, and except for the head, the whole body is

covered with cycloid scales. The mouth of *L. rohita* is inferior and small. Its belly and dorsal side are silvery, and the back is somewhat bluish. The nutritive value of rohu indicated that it is the major source of high-quality protein, and polyunsaturated fatty acids (PUFA) especially omega -3 PUFAs (Memon et al., 2010). In comparison to other carps, it contains the highest percentage of protein (Mukundan et al., 1986) and is a good source of vitamin A, C, and calcium (Roos et al., 2003)

Generally, rohu relies upon its innate immunity as the first and foremost defense mechanism to any pathogen. Furthermore, its adaptive immunity also gets activated whenever any microbe comes in its way to cause infection or harm, etc. (Kar et al., 2015). Ulcer, columnaris, dropsy, icthiophthriasis, argulosis, and epizootic ulcerative syndrome are commonly known diseases of *L. rohita*, mostly caused by bacteria, protozoa, and crustaceans (Farhaduzzaman et al., 2010)

C. catla, commonly known as Thaila in Pakistan is also endemic to riverine systems in Pakistan, India, Bangladesh, Nepal, and Myanmar (Ayyappan and Jena, 2001). It is fast-growing indigenous species of south Asia and popular in aquaculture (Khan et al., 2004; Hussian et al., 2011; Khan et al., 2012). Its head is broad, mouth is wide and upturned, the upper lip is thin and covered by the skin of the snout. The lower lip is moderately thick, broad, and continuous post labial grove. Their eyes are large, and their body with conspicuously large cycloid scales while the head is scaleless (Rahman et al., 2009). The scales on the dorsal side are greyish while whitish on the belly side. Their body is deep and laterally compressed. The dorsal profile is more convex than that of the abdomen. The dorsal side of the body is bluish while silvery on the side, fins dusky (FAO, 2004).

Catla is one of the renowned and fastest-growing carps (Al Mamun and Mahmud, 2014). In carp farming, it is the second most important species after rohu. Generally, Catla grows to 1-1.2 kg in the first year, compared to 700-800 g rohu (FAO, 2013). It reaches up to 182 cm (6.0 ft) long and 38.6 kg (85 lb.) in weight. Its higher growth rate, specific surface feeding habit, and compatibility with other major carps attract culturists to farm, along with *L. rohita* and *C. mrigala* (Pillay, 1990). It is also popular in the six-species polyculture system, i.e., with *L. rohia, C. mrigala, C. carpio, C. idella*, and *H. molitrix*, where it shares the upper feeding niche of the pond with *H. molitrix* (Gjedrem and Baranski, 2009).

Catla is a eurythermal species that grows best at water temperatures between 25-32 °C. The minimum tolerance temperature limit is ~14 °C (Sharma et al., 2016). It attains maturity in its second year and breeds during the summer and rainy seasons like rohu (Lone et al., 2012). It is more difficult to breed in ponds than rohu as it requires more accurate environmental conditions for spawning. Induced breeding perform by hormonal stimulation often gives poor results relative to other major Indian carps (FAO, 2009). Eggs are non-floatable, non-adhesive, and yellowish. Its fecundity generally varies from 100,000-200,000/kg, depending on the fish's length and weight (Chattopadhyay, 2016). Their larvae start to feed three days after hatching (Sheriff and Altaff, 2018). Catla is a surface feeder and omnivorous fish species (Chattopadhyay, 2016). The fry is planktophagous mainly feed on zooplanktons like cladocerans and rotifers. However, adults feed upon zooplanktons along with algal and plant material (Rahman et al., 2009). They can also feed on both natural and supplementary feeds (wheat bran, rice bran, etc.) (Rahman et al., 2009).

Except for growth rate, the consumer preference, market demand, nutritive value, thermal tolerance, and immunity of Catla is less in contrast to rohu (FAO, 2009, 2013; Ahmad et al., 2020). In Pakistan, the price of Catla ranges from Rs 180-350/kg as compared to rohu, and generally, consumers prefer medium-sized fish of 1-2 kg because the taste reduces as fish get larger. Catla has high moisture (77.3%) and ash (1.4%) contents but low crude protein (16.9%) and fat as compared to rohu (Shakir et al., 2013). Similarly, vitamin A, D, E, and K contents are also less than rohu (Paul et al., 2016).

Catla is a sensitive fish (Abhijith et al., 2016) and more susceptible to temperature fluctuation and disease and shows a higher mortality rate generally in early developmental stages as compared to other carps (Iqbal, 2016). The commonly reported diseases affecting Catla include lernaeasis, argulosis (parasitic disease), saprolegniasis (mold infection) (Iqbal, 2016), dropsy (bacterial infection), columnaris (bacterial infection), white spot disease, black spot disease, and gill rot (Rahman et al., 2010).

Both species (*L. rohita* and *C. catla*) have a similar number of chromosomes, i.e., 2n = 50. *L. rohita* contains 8 metacentric, 6 submetacentric, and 4 sub-telocentric, and 32 acrocentric chromosomes while *C. catla* has 6 metacentric, 20 submetacentric, 22 acrocentric, 2 subtelocentric chromosomes (Bhatnagar et al., 2014). Moreover, both species also have overlapping habitats during the spawning season, and probably do not have any

gamete fusion hindrance or developmental block that may inhibits hybridization. Furthermore, in captivity, these carps also spawn synchronously, indicating no major environmental hindrance in human mediating hybridization among these species.

Because of the higher compatibility of both species (*L. rohita* and *C. catla*) for hybridization and variation in quantitative and qualitative traits, several workers carried out artificial hybridization of *L. rohita* × *C. catla*. However, they studied limited traits and reported somewhat variable results. For instance, some investigators observed that the growth performance of the hybrids produced from *L. rohita* ♀ × *C. catla* ♂ cross is better than slower-growing parent *L. rohita* (Varghese and Shantharam 1979; Keshavanath et al., 1980; Reddy and Varghese, 1980; Somalingam et al., 1990; Jana, 1993). However, Chaudhuri (1973) observed that this hybrid grows like *C. catla* and declared it the best hybrid among Indian carps, as it combined the small head of *L. rohita* and the fast growth of *C. catla*. According to Bhowmick et al. (1981), the observed general appearances of the hybrid was intermediate to the parent species, yet possessed more of maternal (*L. rohita*) traits, e.g., flesh content of the hybrid was 54-58% as compared to *L. rohita* (48-52%) and *C. catla* (45%).

Some workers also made a reciprocal cross, i.e., *C. catla* ♀ × *L. rohita* ♂ and reported somewhat higher fertilization percentage and hatching rate and did not find any abnormality during the early development stage (Reddy and Varghese, 1983). The literature revealed the variable growth rate of these hybrids. For instance, some investigators reported the higher growth rate of hybrid, even better than *C. catla* under monoculture and in combination with *C. catla* (Alikunhi et al., 1971; Varghese et al., 1984). However, other workers reported a slower growth rate, i.e., much slower than *C. catla* and slightly slower than *L. rohita, e.g. C. catla* and *L. rohita* showed about 108.9% and 25.2% more weight respectively as compared to a hybrid (Reddy and Varghese, 1980b).

Literature also revealed that the morphometric traits of both *L. rohita* ♀ × *C.catla* ♂ and *C. catla* ♀ × *L. rohita* ♂ hybrids were intermediate to parental species. For instance, morphometric ratios like fish length: head length, head length, snout length, head length: mouth width, and fish length, dorsal fin length was intermediate, and for a ratio of head length to interorbital space, the hybrids were more towards *C. catla*. Furthermore, the head length to the diameter of the eye ratio indicated more resemblance of *L. rohita* ♀ × *C. catla* ♂ hybrid to *C. catla* and *C. catla* ♀ × *L. rohita*♂ to *L. rohita* (Bhowmick et al., 1981;

Jana, 1993). Moreover, both hybrids showed similar gill rakers, and the mouthparts indicate their same food habits (Reddy and Varghese, 1983; Jana, 1993)

In Pakistan, aquaculture is rather a new activity. Regardless of the huge marine, brackish, and freshwater resources (inland water area, 25 220km2), aquaculture production is low could be due to inappropriate aquaculture practices. There is immense potential for the improvement of this sector by adaptating newer trends and technologies. In Pakistan, inland aquaculture is mostly related to freshwater fish farming and consisted of Chinese carps, e.g. silver carp (*H. molitrix*), grass carp (*C. idella*), bighead carp (*H. nobilis)*, Common carp (*C carpio*), and Indian major carps e.g., rohu (*L. rohita*), thaila (*C. catla*) and mrigal (*C. mrigala*) (Ahmed et al., 2008; Shah et al., 2011). These carps are mostly cultured in earthen ponds in a polyculture system by adopting extensive and semi-intensive culture practices. Since 2001, aquaculture production showed a continuously increasing trend and increased rapidly from 57,632 tonnes to reach over 159,083 tonnes in 2018 (FAO, 2019). Moreover, during the past few years, Indian major carps constituted 23% of pond production and 24.6% of total fish production in Pakistan (FRSS, 2010–2011). Despite the improvement in aquaculture production, the contribution of the fisheries sector in agriculture production and GDP is 3.5 % and 1% respectively, indicating a real need to advance the aquaculture sector sustainably.

Although there is a general gap between global supply and demand for fish, however in Pakistan, this difference is much more pronounced and only 2.0 kg/ capita/ annum is available in contrast to America, China, and other countries where it is consumed at the rate of 11-19.8 kg/ capita/ annum (FAO, 2013). Aquaculture can bridge the gap between projected supply and demand for fish. However, this would not be possible without the introduction of a genetic improvement/enhancement program. It is a general view that anyone can boost up carp production in Pakistan by adopting simple genetic techniques like best strain selection, selective breeding, inbreeding, crossbreeding, and hybridization. Many scientists participated in genetic improvement programs of different fish species by using a range of selection methods and provided ample evidence to support the view that fish production can be enhanced by the application of genetic tools (Gjedrem and Baranski, 2009; Nielsen et al., 2010; Shah et al., 2011; Gjedrem and Dunham, 2011; Luo et al., 2014; Haridas et al., 2017).

Since interspecific hybridization is gaining importance in aquaculture and appears as a simple, inexpensive tool to improve the performance of carp, therefore, the present comprehensive study was designed to produce F1 intergeneric hybrids of *L. rohita* and *C. catla* through reciprocal crosses (two purebred cross-types (RR, *L. rohita* × *L. rohita* and CC, *C. catla* × *C. catla*) and two reciprocal hybrid cross-type (RC, *L. rohita* ♀ × *C. catla* ♂ and CR, *C. catla* ♀ × *L. rohita* ♂) and compare the fertilization rate, hatching rate, growth performance, immunity, disease resistance, thermal tolerance, morphometric and phylogenetic relationship of hybrids with parental species. The available literature on a reciprocal hybrid of *C. catla* and *L. rohita* is limited and somewhat controversial and addressed only the growth and morphometry of hybrids. Here, the present study evaluated many traits of both hybrids and parent species by using both biological and molecular markers.

Chapter 1

Production of intergeneric hybrids of *Catla catla* and *Labeo rohita* from reciprocal crosses and evaluation of growth performance

Materials and Methods

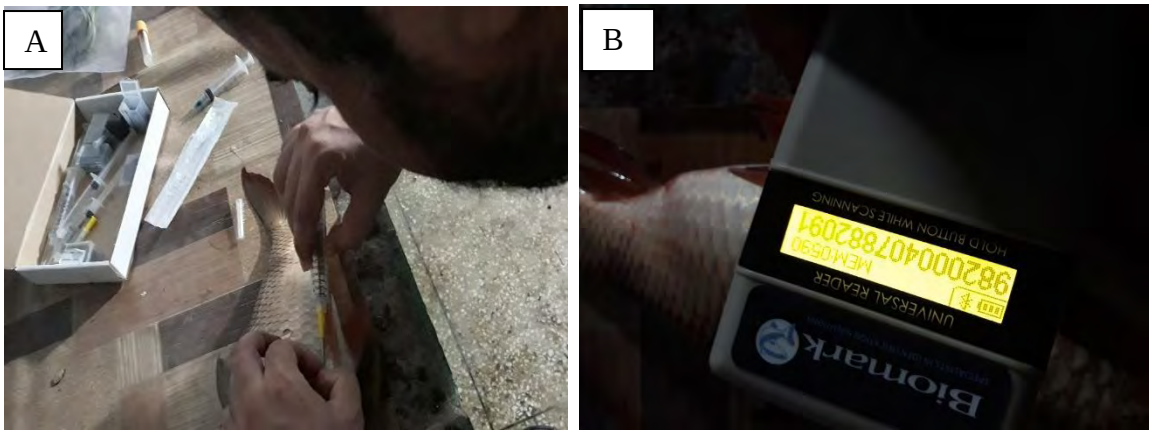
The research was conducted in collaboration with Tawakkal Fish Hatchery & Farm, Muzaffargarh Pakistan**.** The induced breeding of two closely and phylogenetically related species Rohu (*L. rohita*) and Thaila (*C. catla*) was conducted and parent species as well intergeneric hybrids were produced from reciprocal crosses. The comparative morphometric and phylogenetic studies, growth performance, immunity, disease resistance, and thermal tolerance experiments were executed at Fisheries and Aquaculture facility, Quaid-i-Azam University, Islamabad Pakistan. Moreover, Part of molecular work was performed under the supervision of Prof. Isidoro Meton, Department of Biochemistry and Physiology, Faculty of Pharmacy and Food Science, University of Barcelona, Spain by availing IRSIP Program of HEC, while remaining was conducted at home university.

Selection of broodfish

In February 2016, 30-35 healthy adults of each population, with no sign of infection and deformity, body weight rangieng from 2.9-3.3 kg (*L. rohita*) and 3.1-3.8 kg (*C. catla*) were harvested from the main reservoir and shifted to holding tanks for tagging.

Tagging

Fish were held for four days in well-aerated holding tanks for acclimation and then starved for about 30 hrs before inserting Pit Tags with a handheld injection device for identification of individuals. Passive Integrated Transponder is the most popular tag (BIO8. B.03V1 HPT8 Mini Chip) used to mark fish internally. Fish were caught and anesthetized with buffered MS222 (0.1g/L) and, before inserting a PIT Tag into the fish, the implantation site was disinfected with $KMnO_4$. PIT Tag was read by tag scanner (Biomark), and identification code was recorded. Subsequently, a tag was inserted slowly and steadily by using a PIT tag implanter into the muscle above the peritoneal cavity near the pelvic fin of fish. After tag insertion, the site was disinfected again with $KMnO_4$, and fish was released back into the holding tank for recovery. During the initial time of recovery (about 40 hrs after inserting PIT Tag), fish were starved a day and then maintained in well-aerated recovery tanks for about seven days before shifting to well-prepared earthen ponds.



Picture 1. (A) showing Fish tagging and (B) showing reading of identification code

Preparation of broodfish pond

Before stocking selected broodfish, earthen ponds having an area of 0.4 hectares were prepared by following the commonly practiced method (Amir et al., 2020). Briefly, all earthen ponds were drained, sun-dried and their dikes repaired. Afterwards, agricultural lime ($CaCO_3$, 250 kg/ ha) and animal manure (3333.33 kg/ha) was spread on the bottom of all earthen ponds. Initially, ponds were filled with water up to a depth 0.5 m and when pond water become fertile (live feed produced), more groundwater was added and water level was raised to 1.5 m. Fish of both species (*C. catla* and *L. rohita*) were stocked at the rate of 1500 kg/ha. To maintain the fertility of ponds, fortnightly, 1250 kg/ ha of decomposed cow dung (organic fertilizer) along with inorganic fertilizers, urea (31 kg/ha) and triple superphosphate (TSP) (16 kg/ ha) were applied (Rahman et al., 2006). Furthermore, during rearing, daily water level up to 1.5 m was maintained with the addition of fresh water, while ponds productivity was checked occasionally by using a Secchi disc, the disappearance of a disk at a depth <30 cm indicated the restriction of fertilizers.

Rearing of broodfish

Fifteen (15) pairs each of f both species (*C. catla* and *L. rohita)* were stocked in the same earthen pond. The tagged fish at the time of stocking was about three years old. After two days of shifting, fish were provided 32% protein, 6 mm floating pellet feed (Oryza Organic PVT LTD at 2% body weight. Fish reared in ponds for about 3 months before the initiation of breeding experiments. During that period, daily water quality parameters like temperature (˚C), dissolved oxygen (DO level, mg/L), and pH were checked and kept under optimum range, while fertility of the pond was checked with a Sacchi disc and maintained by

the addition of organic (cow-dung) and inorganic fertilizers (urea/DAP). Fresh aerated tube well water was added daily at dawn and dusk to control the oxygen level. Initially, at the stocking time, water temperature was 25°C which increased gradually and reached 28°C, while pH and DO level, although fluctuated but remained within the range suitable for the rearing of Indian major carps (pH 7-8 and DO 5-6 mg/L). Other water quality parameters like hardness (90 mg/L), alkalinity as $CaCO_3$ (80 mg/L) and total dissolved solids (TDS) (185mg/L) etc. were checked fortnightly and did not observe significant fluctuation and were found within a range suitable for freshwater fish culture.

Breeding experiments

Fish breeding experiments were started in June 2016. Briefly, each of 6 ripe dams and 10-12 sires of *C. catla* and *L. rohita* broodfish were harvested and after observing sexual maturity, transferred with care into the hatchery building. For conditioning, they were kept in concrete water holding tanks having well-aerated water. The ripeness of both male and female broodfish was checked by characteristics reported by Metwally et al. (2008) like soft or distend belly with swollen genital opening and smooth pectoral fins for female, while males showed obvious secondary sexual characteristics. Moreover, by slight pressure on the abdomen, the male emitted white color milt.

Induced Spawning

Induced breeding of fish was conducted by following the procedure commonly used for major carps. Briefly, after 2-3 hrs of conditioning, broodfish were weighed individually and injected with a synthetic induced spawning agent, Ovaprim (analog of LHRH + antagonists of dopamine, Syndel, USA) intramuscularly (dose, 0.5-0.7ml/ kg BW) to female broodfish of both species (*L. rohita* and *C. catla*), while males of both species were injected at lower dose i.e., 0.2 ml/kg BW (Mohapatra et al., 2018; Ahmad et al., 2020). After injecting ovaprim, 2 female and 4 male of the same species fish were kept in a concrete circular tank having slow-moving water. For avoiding disturbance, the tank was covered with a canvas screen. Water quality parameters like temperature (˚C) and DO (mg/L) of the tank having ovaprim injected fish were measured frequently after injection to ovulation by using water quality HI-9828 Multiparameter (HANNA Instruments. Inc. Woonsocket, USA), and found within a range between 28-30˚C and 5.2-6 mg/L, respectively, while ammonia was found <

0.25 ppm. For obtaining progeny of similar age, all fish were induced bred to breed on the same day.

Before stripping of female's broodfish, milt of all *C. catla* as well as *L. rohita* was collected separately in test tubes and diluted tenfold (1:9) with 0.9% NaCl. Induced spawning of fish and performed by the method described in detail by Naeem et al. (2013).

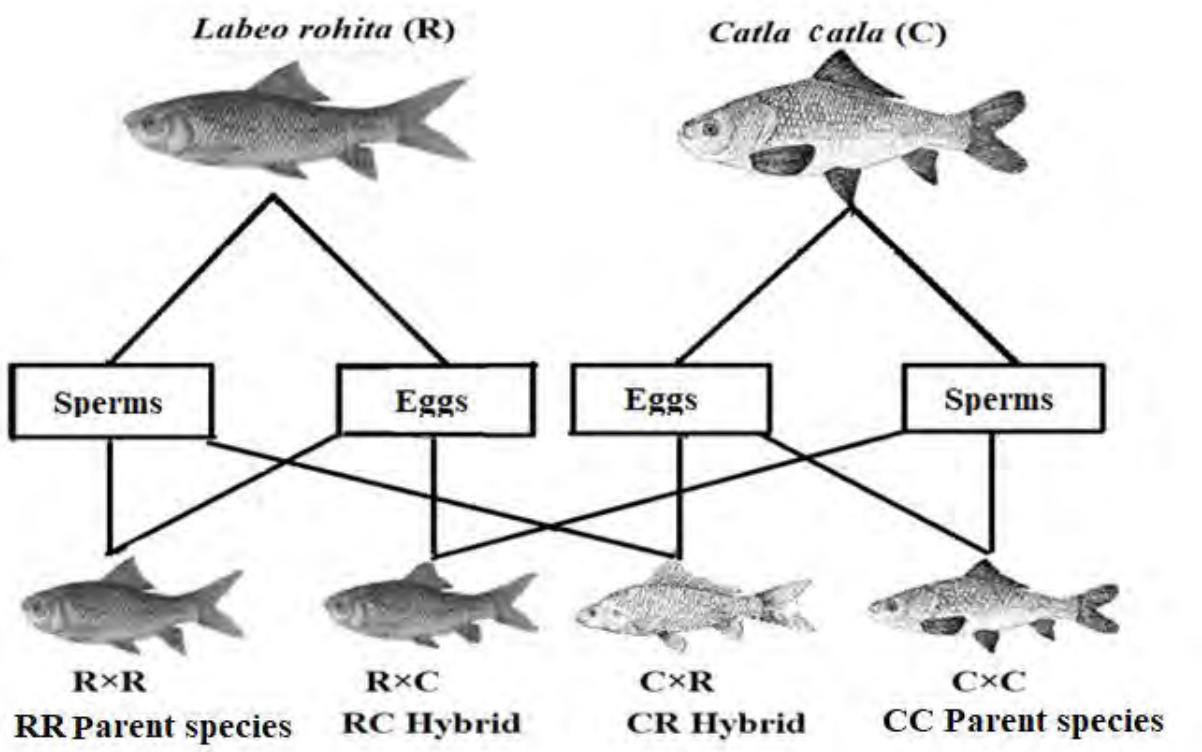


Fig. 1. Four crosses between *L. rohita* (R) and *C. catla* (C), *i.e.*, two purebred cross-types (R× R and C × C), and two reciprocal hybrid cross-types (R × C and C × R)

The first letter designates the dam while the second letter designates the sire (R=*L. rohita*, C= *C. catla*). There are four cross-type codes: RR, RC, CR, and CC

Fertilization

The dry method of fertilization was adopted. Here, reciprocal crosses were made to produce pure species (RR, *L. rohita* and CC, *C. catla*) as well as intergeneric hybrids (RC and CR). Briefly, the eggs of every fish were weighed and equally divided into two groups and

fertilized with sperms of their species and other species respectively. Subsequently, for fertilization, sperm were gently mixed with eggs by using hen feathers for about 3-4 min. After that, tubewell water was added to the mixture of sperm and eggs and constantly stirred for 3-5 min. Afterwards, fertilized eggs were rinsed carefully.. Stirring and rinsing of eggs with water were continuously performed for 2 to 3 times or until eggs became swollen and started to float. Subsequently, the fertilized eggs were shifted to the incubation tank.

Picture 2. Weighing of stripped eggs for enumeration

Egg incubation

In incubation circular tank, water flow rate of 5.0 L/ min was maintained and incubation of fertilized eggs was conducted at ambient temperature.

Percentage fertilization

Fertilization (%) was estimated immediately after transferring fertilized eggs to a circular tank. Briefly, water with eggs was collected at random three times from the centre of the incubation tank in a 500 ml beaker. The eggs in a beaker were calculated and carefully observed. The opaque eggs were considered as fertilized while the transparent as unfertilized eggs respectively. The following formula was used to calculate the fertilization rate.

$$\text{Fertilization (\%)} = \frac{\text{No. of fertilized eggs}}{\text{Total No. of eggs in a beaker}} \times 100$$

Percentage hatchability

The number of eggs hatched from a hundred fertilized eggs is known as percentage hatchability. The time required from the initiation of the incubation process to complete the incubation of eggs was the hatching time. In the incubation tank hatching of eggs was completed in 72 hrs at 29°C. After complete hatching, the total hatchlings were weighed, and a subsample was taken to count the number of hatchlings in the weighed subsample. To determine the total number of hatchlings, the counted hatchlings in the weighed sample were multiplied by the total weight of hatchlings. For calculating the hatching percentage, the following formula was used.

$$\text{Hatchings (\%)} = \frac{\text{No. of hatchlings}}{\text{Total numbers of fertilized eggs}} \times 100$$

Nursing of fry

For 2-3 days after hatching or until the absorption of yolk sac, the yolk-sac larvae were maintained in the incubation tank. Afterward, postlarvae of each cross-type were transferred into separate rectangular tanks and initially fed with prepared live feed (rotifer) and juice of 40% protein powdered feed (Ibrar et al., 2017). Some of these postlarvae were used for evaluating early growth performance while the rest of the stock of each cross-type was stocked separately in nursery ponds.

Experimental design

Early growth performance under controlled conditions

For evaluating growth performance under controlled culture conditions, the experiment was conducted in a replicate of three in fibreglass troughs (capacity; 150 L), well equipped with water heaters for maintaining temperature (27°C) and air stones connected to air pump for DO level (5.6 mg/L). About 600 swim-up fry of each cross, parent species (CC and RR), and hybrids (RC and CR), were randomly selected and equally distributed in their respective 3 tanks (200 fry per tank or 600 fry/ population). To avoid water deterioration, the

once every second days fecal matter/unconsumed feed was removed, and 20% of the water was replaced with fresh water.

Feeding strategy

Fry feed with 40 percent CP level in powder form was purchased from Oryza Organic (Private Ltd.) and provided by adopting the same strategy as reported by Amir et al. (2018) for early rearing of the same species. Briefly, the feeding ratio, frequency, particle size, and CP level were changed with the change in weight and size of the fry. To adjust the feeding rate, every month sampling was done and health status was also observed.

Water Quality

During the rearing period, water temperature (°C), pH and dissolved oxygen (DO, mg/L), were noted twice a day (09:00 hrs and 16:00 hrs) by using water quality HI-9828 Multiparameter (HANNA Instruments, USA), while weekly total ammonia of each tank was checked by using test kit of ammonia for freshwater (HI3824, ROMANIA). Throughout the rearing period, water temperature, DO levels, and pH of all tanks showed minor fluctuations (±0.2°C), while ammonia remained less than 0.035ppm.

Growth performance

After 3-month rearing, fingerlings were starved a day before sampling. On the day of sampling, fish of each population of tank were collected separately, weighed, and counted. For calculating the average body weight (BW) of each fish per population, all counted fingerlings of each tank were divided by the total weight of fingerlings per tank, the value of three tanks/population were pooled and their mean value was calculated for representing average body weight of individual per population. For evaluating growth performance and survival (%), the following standard formulae were adopted.

Wt gain (g) = FBW (fingerlings) – IBW (fry)

$$\text{Wt gain (\%)} = \frac{\text{FBW (fingerlings)} - \text{IBW (fry)}}{\text{IBW (fry)}} \times 100$$

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

Average daily weight gain (ADG) = FBW −IBW/ rearing period (days)

Survival rate (%) = Ni – Nf/ Ni × 100

Where

Wt = Weight

IBW= Initial body weight

FBW = Final body weight

ln= Natural log

SGR = Specific growth rate

Nf = Number of fingerlings at the end of the rearing period

Ni = Number of postlarvae at the time stocking

Heterosis

Hybrid vigor or the improved growth of both F1 hybrids (CR and RC) over the average of the parents were calculated by following standard formulas

$$\text{Low parent heterosis (LPH)} = \frac{F1 - P_{low}}{P_{low}}$$

Mid parent heterosis (MPH)= $\frac{F1 - MP}{MP}$

High parent heterosis (HPH) = $\frac{F1 - HP}{HP}$

Where F1 = hybrid

P= parent

Early growth performance under semi-intensive culture system

For evaluating the growth performance of postlarvae in earthen nursery ponds under a semi-intensive culture system, an experiment was conducted in a replicate of three for 3 months (mid-June to mid-August)

Nursery pond preparation

All nursery ponds (size; 120 m^2 each) were earthen and rectangular having an average depth of 1.3 m. They were supplied with groundwater from a tube-well. Before initiation of the experiment, all ponds were sun-dried and aquatic vegetation on the bed was removed and their dikes were repaired. All nursery ponds were fertilized and live feed was produced by the method mentioned above. Initially, nursery ponds were half-filled with water and when water became fertile, i.e., about one week later, with the addition of water, filled the pond up to 1-1.2 m. During the experiment, the water level was maintained by adding fresh water at dawn and dusk and pond productivity by the application of organic and inorganic fertilizers. Occasionally, ponds productivity was checked with the help of Sachi disc, reading at a depth <30 cm indicated the restriction of fertilizers.

Stocking

Early rearing of each cross-type was conducted in a replicate of three (3 tanks per hybrid cross) by adopting a commonly used method for early rearing of Indian major carps. Briefly, 60,000 postlarvae (after yolk sac absorption) of each cross-type were taken from the incubation tank, by using the volumetric method and stocked separately in nursery ponds, having an area of 0.10 hectares. The stocking density of the larvae was 2500 /decimal (Shah et al., 2011)

Feeding strategy

Fry were provided 40 percent protein feed in powder form as a supplementary diet. Feed was purchased from a local feed mill (Oryza Organic, PVT, Ltd), and fed daily in 2 feeding regimes. The feeding ration and CP level were changed with the change in fish weight and size. Initially, during the first week, 40% CP feed was given at the rate of 50 percent of the biomass and then gradually decreased to 25 percent of biomass and end with a 35% CP diet at the rate of 7 percent of the biomass. Sampling was done every month and adjusted the feeding rate.

Water Quality

During rearing, water temperature (°C), pH and dissolved oxygen (DO, mg/L) were noted twice a day (09:00hrs and 16:00 hrs) and total ammonia weekly by using the above-mentioned water quality Multiparameter and test kit of ammonia for freshwater. At the time of stocking, the water temperature of the entire nursery pond was 27.5ºC. It slowly and gradually increased during the rearing period and reached 29°C while the DO level fluctuated somewhat but remained between 5 and 6 mg/L. The total ammonia of all tanks remained at < 0.035 ppm. All the nursery ponds were adjacent to each other and had similar aeration and water supply system, therefore the water quality parameters of different ponds were almost similar and within the range suitable for the rearing of Indian major carps.

Growth performance

After 90 days rearing in earthen ponds, fingerlings were starved a day and on the day of sampling, ponds were drained, and fingerlings of each pond were collected, weighed and

the total number of fingerlings per tank was noted for estimating mean survival (%) and other growth performance indices of each cross-type by adopting standard formulae indicated above

Growth performance of fingerlings in solitary

After observing growth performance, Fingerlings of each cross-type from nursery ponds were shifted to eight already prepared earthen rearing ponds (two/cross-type) at a stocking density of 5000 fish/ ha (Gjerde et al., 2019). Each pond was 0.04 ha and adjacent to each other.

Feeding strategy

Pakistan has a continental type of climate with seasonal temperature variation. The water temperature in most of the areas of Punjab starts to decline in September and reach the minimum value in January and then gradually rises and shows peak values during June-July. Therefore, during the rearing period, extreme weather conditions i.e., thunderstorm, rainy/cloudy season and extreme daily variation in water temperature were observed and we adjusted the feeding rate accordingly. Feeding was restricted during the rainy or cloudy condition and at low temperature, the limited feed was provided because, in this condition, most of the fish do not feed on floating pellets and depends on naturally occurring feeds in the pond waters (Ramakrishna, 2013). The same feeding strategy was adapted for all cross-types

Water Quality

The water quality parameters of each pond were checked by adopting the same strategy as mentioned above. At the time of stocking, water temperature and DO level was 25.5˚C and 5.9 mg/L respectively. During rearing, water temperature and DO level showed fluctuation and ranged between 25.5- 17˚C, DO, 5.7-6.3 mg/L, while pH ranged between 7.6-7.8. Lastly, the ammonia level (<0.025 mg/L) did not show any noticeable changes during the study period.

Growth performance

During the rearing period, water temperature reached the minimum value and showed great variation, therefore, we did not further disturb the fish and reared for about eight months. After that, fingerlings were starved a day and on the day of sampling, about 150-200 fingerlings /pond was harvested for estimating growth performance indices by adopting standard formulas reported above.

Growth performance of Fingerlings in communal ponds

Fin clipping for stock identification

Fingerlings of RC and CR hybrids with bodyweight range 23-26g were harvested from their respective ponds and shifted to holding tanks. Each stock was kept in a separate tank. For clear and quick identification, the right pectoral fin of RC hybrids and left pectoral fin of CR hybrids were clipped and $KMNO_4$ was applied on the clipped site. Fingerlings were remained in holding tanks under well-aerated water for about 7 days before shifting to earthen ponds. Moreover, during that period, they were closely observed for any infection.

To evaluate the comparative growth performance of fingerlings, two sets of experiments were conducted one under control conditions in rectangular fiberglass tanks (500 gallons) and the other in earthen ponds by adopting a semi-intensive culture system.

Rearing in a communal tank under controlled conditions

Communal rearing of all cross-types in controlled conditions was conducted in fiberglass tanks, from May to July. For this experiment,75 fingerlings of each cross-type, parental species (RR, CC) and hybrids i.e., RC hybrid (right pectoral fin-clipped) and CR hybrid (left pectoral fin-clipped) having no sign of infection or disease symptoms were randomly stocked in 3 fiberglass tanks (100 fingerlings/ tank, 25/cross-type) at a stocking density of about 2.0 g/L. Tanks were supplied with the aerators and water heaters to maintain the dissolved oxygen level (6.0mg/L) and temperature (27˚C). After shifting, fingerlings were starved a day and then feeding started.

Feeding strategy

Fingerlings were fed with 35% protein commercial 2 mm pellet feed (Oryza Organic Pvt, Ltd), at the rate of 5 percent body weight daily in two feeding regimes. The feeding rate and size of the pellet were changed with the change of fish weight and size and it was ended with a 3% body. The feeding rate was modified fortnightly by observing the weight of random samples.

Water Quality

Water quality parameters during rearing were monitored by the same strategy as mentioned above. Rearing of fish was conducted in controlled conditions, therefore water quality parameters showed very small fluctuations. Furthermore, after every day, fecal matters were removed through manual siphoning and about 10-20% water of each tank was exchanged with freshwater. This practice was adapted to avoid water deterioration and to maintain the quality of water.

Growth performance

After 3 months of rearing under controlled conditions, feeding was restricted for 24 hrs before sampling. On the day of sampling, fish of each cross-type were collected and weighed. For evaluating growth performance, the standard formulas mentioned above were adopted.

Rearing in a communal pond under a semi-intensive culture system

For evaluating their growth performance in semi-intensive culture system (a common practice in Pakistan), all cross-types fingerlings were stocked in the same earthen pond (communal pond). Rearing experiment was conducted in triplicate, i.e., in 3 communal ponds. Before stocking, three rectangular earthen ponds (size 0.04ha), adjacent to each other were prepared by the method mentioned and 300 fingerlings of each population (parent species and their intergeneric hybrids) were evenly stocked in three earthen ponds at the rate of 75 fingerlings/cross-type/pond. During rearing, the water level of each pond was

maintained with the addition of water while pond productivity was checked with sacchi disk and maintained by using cow-dung and inorganic fertilizer (DAP or Urea).

Feeding strategy

Fingerlings were fed with 32% CP pellet feed of 2mm size purchased from Oryza Organic (Pvt. Ltd). Daily, they were provided at the rate of 3% body weight in two feeding regimes i.e., about two-thirds of the feed was given in the morning (8:00 hr), while the rest in the afternoon (16:00 hrs). The feeding ratio and CP were changed with the change of fish weight and size. Initially, the pellet size was 2mm with 32% protein but ended with a 4mm pellet having 25% CP. Fortnightly, random sampling was done and the feeding rate was adjusted according to the weight of fish.

Water Quality

During rearing, water quality parameters were checked routinely by adopting the same strategy as mentioned before. Water temperature at the time of stocking was 26.5˚C while the DO level was 5.9 mg/L. During rearing, water temperature, DO level and pH showed fluctuation and ranged between 26.0- 30˚C, 5.1-6.3 mg/L and 7.6-8.7 respectively, while ammonia remained <0.035 mg/L.

Growth performance

After 3 months of rearing, fish were starved a day and on the day of sampling, fish of all cross-types were harvested. Fish of each cross-type/tank were weighed and their number was counted for the evaluation of survival rate and growth performance. All fish except 3 /cross-type/pond was released back in the pond. For evaluating growth performance, above mentioned standard formulas were adopted.

Gene Expression of MyoD and Myogenin

For evaluating the expression of genes related to the growth of skeletal muscle (MyoD and Myogenin and IGF-1), the separated 3 fish of each cross-type per tank (9 fish /cross-type) were dissected and approximately 50 mg each of muscle and liver per fish, were

taken aseptically in RNAlater (50 mg/ml). Samples were stored at -20°C until they were used for RNA extraction.

RNA Isolation

Tissue samples were taken out from RNAlater and thawed on ice for 10-15 min. Subsequently, a 50 mg sample was homogenized in microcentrifuge tubes with the aid of pellet pestles and added 500µL of ice-cold TRIzol® Reagent. The resultant homogenate was incubated at ambient temperature for 5 min. Subsequently, with the addition of 100 µL, the homogenate was agitated vigorously for about 15sec and again incubated at ambient temperature for 5 min followed by centrifugation at 12,000 rpm at 4°C for 15 min. After centrifugation, the upper aqueous layer was transferred to a fresh microcentrifuge tube by using a wide bore micro-tip. Subsequently, with the addition of chilled isopropyl alcohol (absolute) vortex briefly and then the sample was incubated for 10 min at ambient temperature. Afterwards, the sample was centrifuged at 12,000 rpm at 4°C for 10 min and then the liquid phase was discarded and saved in the pellet. The pellet was washed twice with 75% ethanol (500µL) prepared in DEPC treated water. After air drying, the pellet was dissolved in 50 µL of nuclease-free water (Hi-media, India). The isolated RNA was stored at low temperature (-80°C) till further analysis.

RNA Quantification

The quality i.e., purity of isolated RNA and quantity (concentration) of RNA in the sample were accessed by using NanoDrop^{TM} (ND-1000, UV-visible spectrophotometer, Thermo Scientific, USA). For quantification, NanoDrop was set at λ 260 nm and sample absorbance was noted, while for checking the purity of the samples two readings one at λ 260 and other at λ 280 were taken and then the ratio of OD at both wavelengths (260nm and 280 nm) was calculated for observing the quality of RNA in the sample with expected values between 1.9 and 2.0.

cDNA Synthesis

Amir et al. (2019) method was adopted and isolated RNA of each sample was reverse transcribed to cDNA. Briefly, 20 µL reaction mixture was prepared by mixing 8µL RNA, 4µL buffer, 1ml each of dNTPs, 1µL of MMLV-RT, 2.5 µL Random primers,0.5 µL (RNasin® Ribonuclease Inhibitor) and 3µL DEPC water. Subsequently, the reaction mixture was kept in a water bath set at 37˚C and incubated for an hr followed by 5 min incubation at 55˚C in PCR Machine (BIO-RAD T100™ thermal cycler). The concentration of synthesized cDNA in each sample was assessed by using Nanodrop ND-1000 (Thermo Scientific, USA). The samples were stored at −20 °C

Designing of Primers

The primers used for the study were self-designed by using Oligo Primer Analysis Software version 1.1.2 based on the mRNA sequence of the respective genes of *L. rohita* obtained from gene bank NCBI (www.ncbi.nlm.nih.gov). The designed primers were manufactured from Humanizing Genomics Macrogen. The specificity of each primer was checked by running a simple PCR reaction and performing gel electrophoresis, while the quality of cDNA and its compatibility with primers were checked by performing PCR of each sample in duplicate in at least two independent runs. A list of primers used for evaluating the expression of growth-related genes Myogenin, MyoD and IGF-1 are given in Table 2.

For amplification reaction, 15µL reaction mixture was prepared by mixing 0.5µL cDNA, 0.3 µL each of reverse and forward primers, 0.3 µL dNTPs, 0.09µLTaq polymerase 1.5 of 10X PCR buffer and 12µL PCR water (Sure Bio-Diagnostic and Pharmaceutical). The PCR reaction was conducted by using gradient thermal cycler PCR (BIO-RAD T100™ thermal cycler). The PCR process consisted of initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 sec, then annealing at lower temperature i.e., at 55°C for 30 sec followed by extension at 72°C for 2 min. The final step consisted of elongation at 72 °C for 5 min followed by a hold at 4°C. To make sure that the PCR reaction yield adequate amplicon sizes, electrophoresis of PCR products was performed and visualized on 1 % agarose gel containing ethidium bromide.

RT - qPCR

For evaluation of the relative expression of growth-related genes Myogenin, MyoD and IGF-1 in the liver and muscle of all cross-types, quantitative real-time PCR was conducted. Initially, PCR conditions and cycle number were optimized for every studied gene. Each RT-qPCR reaction was run in the standard of 20 µl reaction mixture having 1.6 µl of diluted cDNA, 0.4 µl of forward and reverse primer, 10 µl of SYBER green and 7.6 µl of H_2O.

RT - qPCR reaction condition included an initial denaturation at 95°C for 10 min followed by 40 cycles at 95°C for 15 sec and subsequently, 62˚C for 15 sec. The 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 samples. The mRNA levels of MyoD, Myogenin, and IGF-1 were normalized to the expression of *L. rohita and C. catla*18-s Housekeeping genes, primers are shown in Table 2. The PCR was run in duplicate while each sample was run in triplicate in each PCR. The relative variation in gene expression was calculated by the standard ΔΔCT method (Pfaffl, 2001)

Table 1. Reaction mixture for simple polymerase chain reaction

Reagents	Volume (µL)
cDNA	0.5
Forward primer	0.3
Reverse primer	0.3
10× buffer	1.5
dNTPs	0.3
Taq polymerase	0.09
PCR water	12.01
Total reaction volume	15

Table 2. Primers of housekeeping gene 18s and three growth related genes Myogenin, MyoD and IGF-1 with their optimum temperature, amplicon size and accession number

Target Genes	Primer	TEM.	Amplicon Size	Accession No
Myogenin	F<AGGCGGCGATAACTTCTTCC	66.4		
			228	KR:560074.1
	R<TCCGTTTGACAGCAACCTTC	65.2		
MyoD	F<GCTTTCGAGACCCTCAAGAG	63.0		
			211	KC:344537.1
	R<AATCCATCATGCCATCAGAG	62.5		
	F<GTGCACCCGTAAAGCCCG	68.8		
IGF-1			158	Kx455870.1
	R<TCTGCCCCCTGTGTTTCCTC	68.7		
	F<CGGACACGGAAAGGATTGAC	66.6		
18s			84	KC:915025.1
	R<CGCTCCACCAACTAAGAACG	64.6		

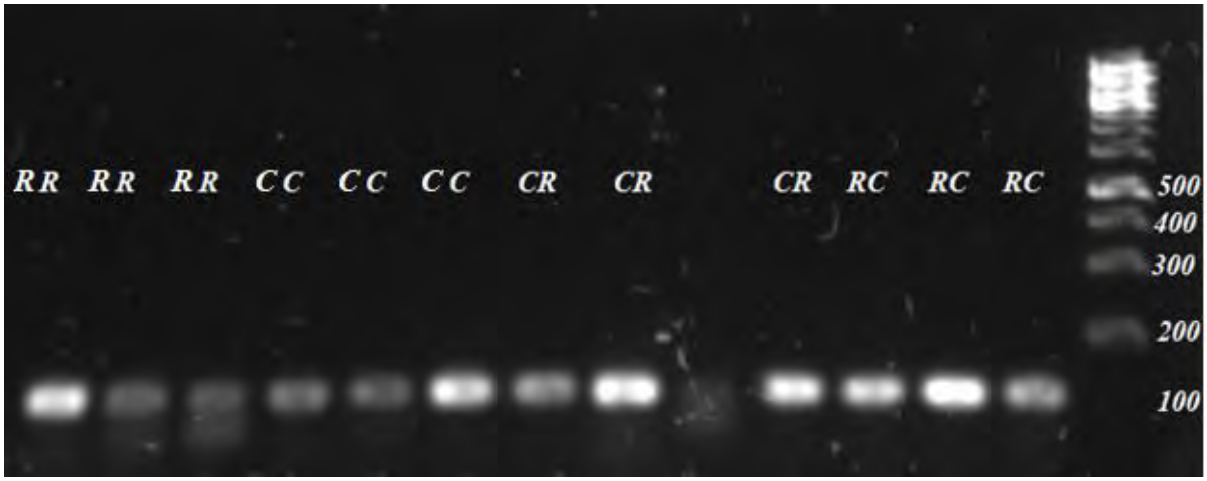


Fig. 2. Confirmation and verification of amplicon size of cDNA

RR= R♀ × R♂, CC= C♀ × C♂, CR = C♀ × R♂ and RC= R♀ × C ♂

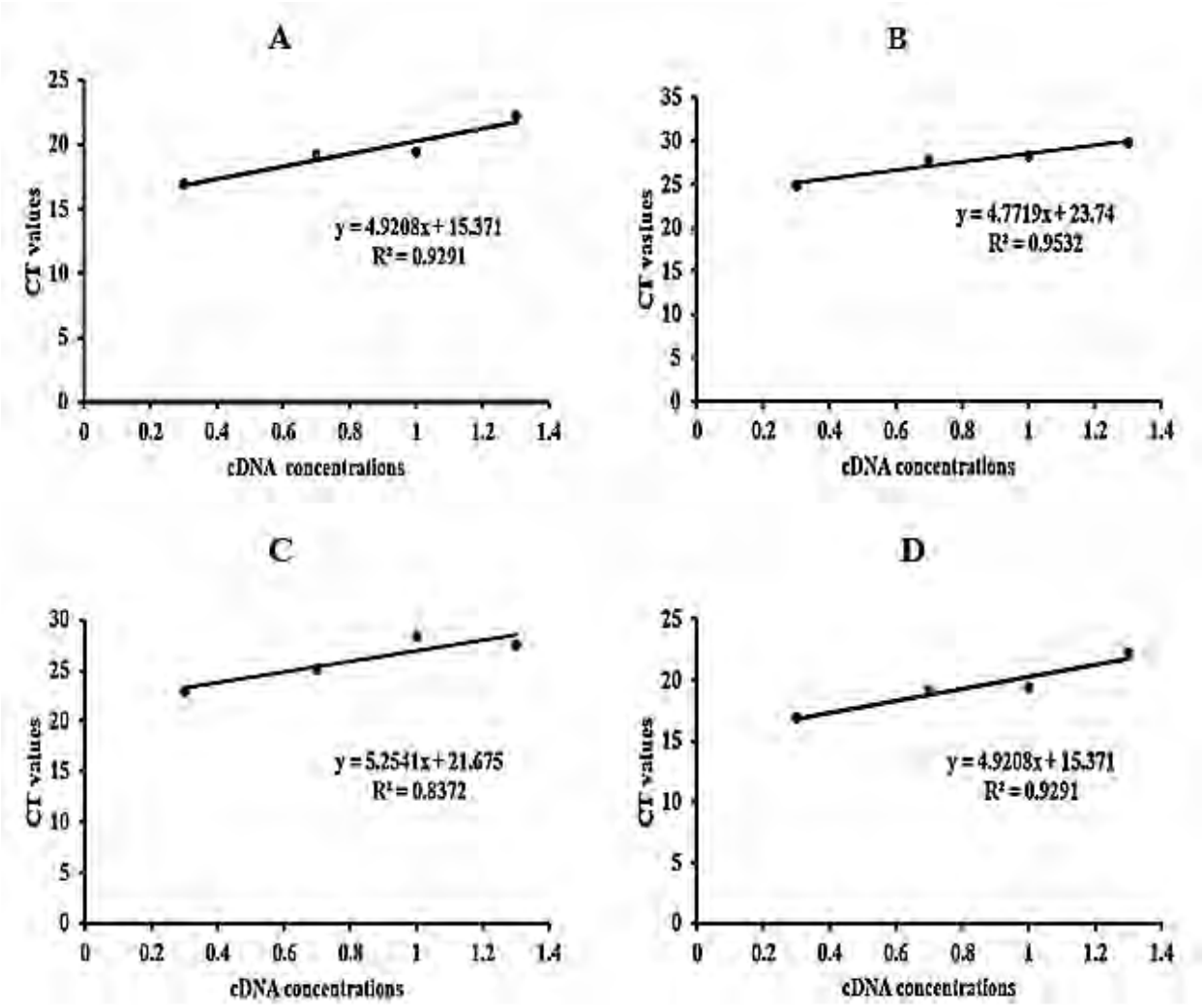


Fig. 3. Efficiency of primers of respective genes (A) housekeeping gene 18s (B) Myogenin (C) MyoD, (D) IGF-1

Statistical analysis

All data were represented as Mean (± SE). Statistical analysis was conducted using computerized SPSS data analysis software version 20 (Chicago, USA) and Statistic version 8.1. By using statistical software, One-way analysis of variance (ANOVA) was performed to determine any potential significant difference among the means of growth, immunity, disease resistance indices, thermal tolerance, etc. indices of different cross-types, while Two-way ANOVA was used to find out spatial and temporal significant difference among means of mRNA levels of growth-related genes of different cross-types. For all pairwise comparisons between the means of indices of all cross-type, after ANOVA, a post hoc least significant difference (LSD) test was applied. Moreover, before running any statistical analysis, Bartlett and Shapiro-Wilk's tests were used to assess data for variance homogeneity and normality distribution. The mRNA levels of growth-related genes were determined by adopting a relative quantification method (Pfaffl 2001). For statistical evaluation of all results, a significance level (α) 0.05 (5%) was set. For a graphical representation of results, GraphPad Prism 5 software was used, and data were plotted as mean values along with their standard error.

Results

Fertilization rate (%)

Statistical analysis revealed a significant difference in the fertilization rate among cross-types (n=9, ANOVA, $F_{3,32}$= 30.53, P<0.001, Table 3). The post hoc all pairwise comparisons showed significantly higher and statistically comparable fertilization rate of both purebred cross-types (RR and CC) as compared to hybrid cross-types (CR and RC). Furthermore, both hybrid cross-types also showed statistically similar fertilization rate

Hatching rate (%)

Like fertilization rate, statistical analysis revealed a significant difference in the % hatching rate (n=9, ANOVA, $F_{3,32}$= 19.87, P<0.001, Table 3) among all cross-types. All pairwise comparison showed statistically comparable and significantly higher % hatching rate of both parent species cross-types (RR and CC) as compared to both hybrids cross-types (CR and RC). Furthermore, both hybrid cross-types also showed a statistically similar % hatching rate

Growth performance

Early growth performance under controlled conditions

Early growth performance of all cross-types under controlled condition is presented in (Table 4). Statistical analysis revealed significant difference in final weight (n=3, ANOVA, $F_{3,8}$= 60, P<0.001), weight gain (n=3, $F_{3,8}$= 61.5, P<0.001), weight gain % (n=3, $F_{3,8}$= 32.2, P<0.001), SGR% (n=3, $F_{3,8}$= 28.1, P<0.001) and gain in biomass (n=3, $F_{3,8}$= 11.2, P = 0.003) among cross-types. The post hoc all pairwise comparison indicated the highest final weight, weight gain % and SGR% of CC followed by CR hybrid while RR showed the lowest values. The pairwise comparison among hybrids indicated considerably (p<0.05) higher weight gain and SGR% of CR hybrid (Table 4). For growth performance parameters, both F1 hybrids showed low parent heterosis, while only CR hybrid had shown favorable mid parent heterosis (Fig. 8). Furthermore, a significant effect of the dam on growth performance indices of hybrids was observed.

Early growth performance in semi-intensive culture system

The postlarvae of all cross-types showed significantly different growth performance in semi-intensive culture system (Table 5). Statistical analysis indicated a significant difference in final weight (n=3, ANOVA, $F_{3,8}$ =127, P<0.001), weight gain (n=3, ANOVA , $F_{3,8}$ =30.8, P<0.001), weight gain % (n=3, ANOVA, $F_{3,8}$ =9.53, P=0.005) and SGR (n=3, ANOVA, $F_{3,8}$ =9.55, P=0.005) among postlarvae of all cross-types after 90days rearing. The post hoc range test indicated a similar trend as observed under controlled conditions, i.e., CC followed by CR hybrids showed significantly higher values of growth indices (final body weight, weight gain % and SGR %) as compared to others (RR and RC hybrid). The pairwise comparison among hybrids also showed improved growth performance of CR hybrid in contrast to RC hybrid.

The growth performance of both hybrids in comparison to parents was intermediate. However, CR hybrids showed relatively higher low parent heterosis as compared to RC hybrid (Fig 8). Moreover, weight gain and SGR% of CR hybrids showed favorable heterosis (MPH= 8 and >2% respectively) while RC hybrid demonstrated unfavorable heterosis for weight gain (MPH= -2.81%).

Growth performance of fingerling in solitary

Fingerlings of all cross-types showed significant variation in the growth performance indices (Table 6). Statistical analysis revealed considerable difference in final weight (n=3, ANOVA, $F_{3,8}$ =22.11, P<0.001), weight gain (n=3, $F_{3,8}$ = 30.76, P<0.001),weight gain % (n=3, $F_{3,8}$=9.53, P<0.005) and SGR% (n=3, $F_{3,8}$ =9.55, P<0.005) among fingerlings of all cross-types. The post hoc LSD pairwise comparison indicated the highest final weight, weight gain % and SGR % of CC followed by CR hybrid while RR showed the lowest values. The pairwise comparison among hybrids indicated significantly ($p<0.05$) higher weight gain and SGR % of CR hybrid (Table 4). The growth performance of both hybrids in comparison to parents again was intermediate. However, CR hybrids showed relatively higher LHP and positive MPH as compared to RC hybrid. Both F1 hybrids had shown low parent heterosis for growth related parameters (Fig 9)

Growth performance of fingerling in communal tank in controlled conditions

At the end of 90 days of rearing in controlled conditions, F1 fingerlings of all cross-types showed significantly different growth performance. Statistical analysis revealed significant difference in the final body weight (n=3, $F_{3,8}$ =90.53 P<0.001), weight gain (n=3, ANOVA, $F_{3,8}$ =24.29, P<0.001), weight gain % (n=3, $F_{3,8}$ = 0.629, P<0.616) and SGR% (n=3, ANOVA, $F_{3,}$8 = 0.691, P<0.583) among fingerlings of all cross-types. The Post hoc LSD test indicated significantly higher weight gain % and SGR% of CC followed CR hybrids, while RR and RC hybrid showed significantly (p<0.05) lower but statistically similar (P>0.05) values of growth indices as compared to CC and CR hybrids (Table 7). Again, the growth performance indices of both F1 hybrids in comparison to parents were intermediate and a significant effect of the dam was observed. Furthermore, CR hybrid showed many-fold higher LPH and positive MPH as compared to RC hybrid (Fig 10).

Growth performance of fingerlings in communal pond under intensive culture system

Fingerlings of all cross-types after 90 days rearing in communal pond in a semi-intensive culture system showed significant difference in the final weight (n=3, $F_{3,8}$ = 674.28, P<0.001) weight gain (n=3, $F_{3,8}$=663.78, P<0.001) SGR% (n=3, ANOVA, $F_{3,8}$ =39.21, P<0.001), weight gain % (n=3, ,$F_{3,8}$ =33.85, P<0.001) and gain in biomass (n=3, $F_{3,8}$ = 664, P<0.001). The Post hoc LSD test revealed considerably higher values of growth related indices of CC followed by CR hybrids, while RR showed significantly (p<0.05) the lowest values. Moreover, the CR hybrid had shown significantly (p<0.05) higher values of growth performance indices as compared to RC hybrids (Table 8). Both F1 hybrids had shown heterosis for growth performance parameters (weight gain and SGR). However, CR hybrid demonstrated a higher value of heterosis for body weight (LPH= 66.92% and MPH=14.8%) as compared to RC hybrid (LPH = 44% and MPH= -0.97%).

Relative Myogenin gene expression

After 90 days of rearing in the communal pond under a semi-intensive culture system, fingerlings of all cross-types revealed a significant difference in the mRNA levels of growth-related gene Myogenin in the liver (n=9, Two-way ANOVA, $F_{3,72}$=62.98, P<0.001) and muscle (n=9, $F_{1,72}$=331.02, P<0.001). Furthermore, a significant interaction between cross-

types× tissue (n=9, $F_{3,72}$=49.20, P<0.001) indicated the manner how the gene showed tissue specific expression. The Post hoc all pairwise comparisons showed significantly higher (P<0.001) Myogenin gene expression in the CC followed by F1 CR hybrid while the lowest expression was observed in RR. Furthermore, in the fingerlings of all cross-types, Myogenin expression was significantly (P<0.001) low in the liver in contrast to the muscle. (Fig 5). Both hybrids demonstrated a higher level of favorable LPH and unfavorable MHP for the expression of Myogenin in both tissues. However, CR hybrids showed higher level LPH (liver, 180.52%; muscle, 259.43 %) as compared to RC hybrid (liver, 79.50%; muscle, 94.77 %) (Fig 5).

Relative MyoD gene expression

Relative expression of MyoD in the liver and muscle of fingerlings of all cross-types is shown in (Fig. 6). Statistical analysis also indicated a significant difference in the expression of growth-related gene MyoD in the liver and muscle (n=9, Two-way ANOVA, $F_{1,72}$=196.71, P<0.001) of all cross-types (n=9, Two-way ANOVA $F_{3,72}$=50.90, P<0.001). Furthermore, a significant interaction between cross-type× tissues (n=9, $F_{3,72}$=21.68, P<0.001) indicated the manner how the gene showed tissue specific expression in the fingerlings of all cross-types. The Post hoc all pairwise comparison test revealed significantly (P<0.001) higher MyoD gene expression in the CC followed by F1 CR hybrid while the lowest expression was observed in RR. Furthermore, in all cross-types, MyoD gene expression was significantly lower (P<0.001) in the liver compared to the muscle. Both hybrids demonstrated favorable LPH for the expression of Myogenin gene in both tissues. However, CR hybrids showed a higher level of LPH (liver, 164.67%; muscle, 280.52 %) as compared to RC hybrid (liver, 56.05%; muscle, 106.55 %). The estimate of MPH for the expression of MyoD ranged from -13.94 in the liver to 10.56% in the muscle of CR hybrid (Fig 6)

Relative IGF-1 gene expression

Like other growth elated genes, the relative expression of the IGF-1 gene was also significantly different in the liver and muscle (n=9, $F_{1,72}$=73.55, P<0.001) of all cross-types (n=9, $F_{3,72}$ =100.86, P<0.001). Furthermore, strong interaction between cross-types × tissues (n=9, $F_{3,72}$=12.14, P<0.001) demonstrated the way how both variables influenced the

expression of the IGF-1 gene. The Post hoc all pairwise comparison test showed significantly higher ($P<0.001$) IGF-1gene expression in the CC followed by CR hybrid while the lowest expression was observed in RR. Moreover, the pairwise comparison among tissues indicated significantly higher expression in the liver of all cross-types in contrast to muscle. (Fig 7). Both hybrids demonstrated favorable LPH for the expression IGF-1 gene in both tissues. However, CR hybrids showed a higher level of LPH (liver, >274 %; muscle, >167 %) as compared to RC hybrid (liver, 107.96%; muscle, 76.65 %). The CR hybrid also showed positive MPH for the expression of the IGF-1 gene in the liver (>23%) and muscle (>21%) as compared to the RC hybrid which showed unfavorable MPH (liver, -31.38%; muscle -19.90%) (Fig 7).

Table 3. Fertilization rate (%) and hatching rate (%) of all cross-types

	Cross-types			
	Purebred		Hybrids	
Parameters	**RR**	**CC**	**CR**	**RC**
Fertilization rate (%)	93±0.80[a]	91.22±1.43[a]	82.67±0.53[b]	84.56±0.58[b]
Hatchings rate (%)	87.22±0.89[a]	86.44±1.53[a]	78.78±0.83[b]	79.99±0.70[b]

Data presented as Mean (± SE). Sample size (n) =9 for each cross-type. All means values having different lowercase superscript within a row are significantly different ($P<0.05$). RR= R♀ × R♂, CC= C♀ × C♂, CR = C♀ × R♂ and RC= R♀ × C ♂.

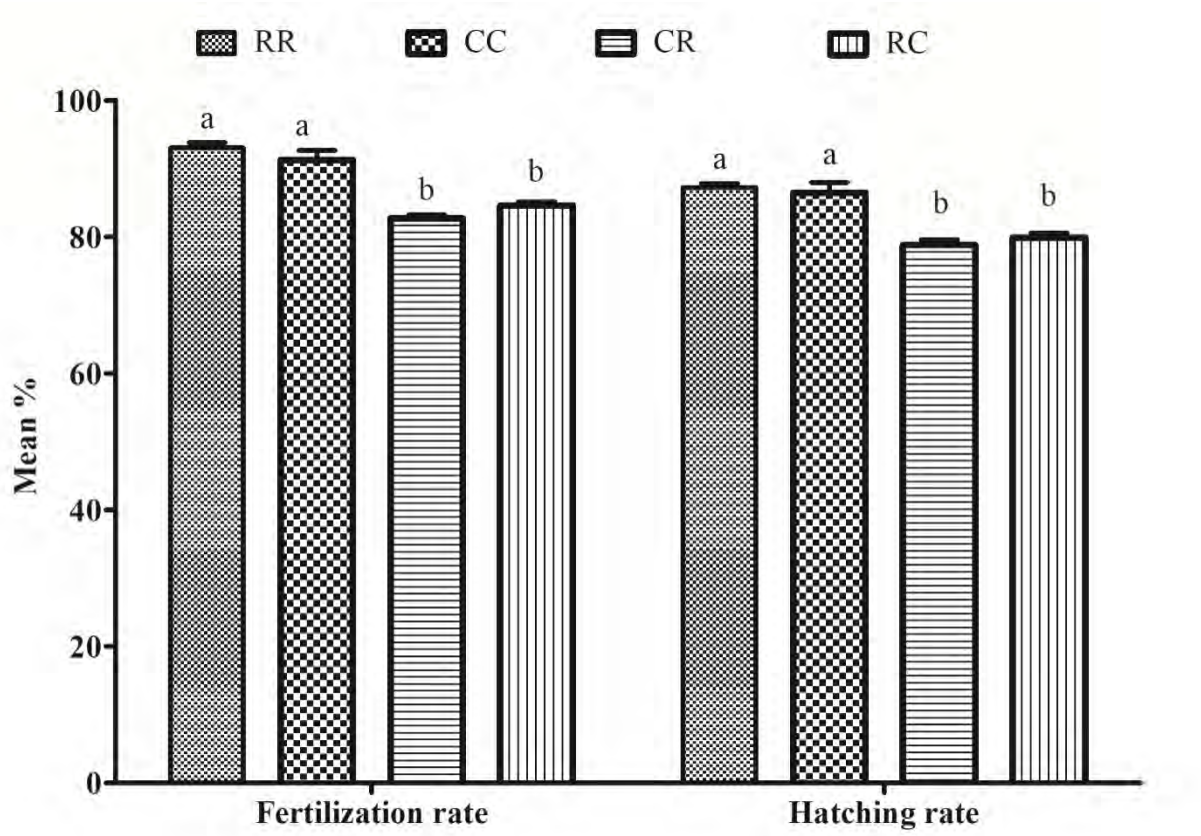


Fig. 4. Mean (± SE) fertilization rate and hatching rate of all cross-types. Sample size (n) = 9 for each cross-type. All means having different lowercase superscripts on the bar are significantly different (P< 0.05). RR= R♀ × R♂, CC= C♀ × C♂, CR = C♀ × R♂ and RC= R♀ × C ♂.

Table 4. Early growth performance of postlarvae of all cross-types after 90 days rearing in the indoor facility under control conditions

	Cross-types			
	Purebred		Hybrids	
	RR	**CC**	**CR**	**RC**
IBW (g)	0.27 ± 0.06^{b}	0.30 ± 0.09^{a}	0.28 ± 0.01^{ab}	0.28 ± 0.02^{ab}
FBW (g)	3.18 ± 0.25^{d}	5.26 ± 0.24^{a}	4.83 ± 0.40^{b}	3.75 ± 0.27^{c}
Wt. gain (g)	2.91 ± 0.14^{d}	4.96 ± 0.08^{a}	4.55 ± 0.08^{b}	3.48 ± 0.16^{c}
Wt. gain % (g)	1106.13 ± 52.80^{c}	1678.15 ± 63.17^{a}	1626.19 ± 31.49^{a}	1288.95 ± 39.06^{b}
SGR (%BW /day)	2.64 ± 0.02^{c}	3.01 ± 0.04^{a}	2.96 ± 0.02^{a}	2.78 ± 0.03^{b}
ADG (g)	0.02 ± 0.01^{a}	0.03 ± 0.01^{a}	0.03 ± 0.01^{a}	0.02 ± 0.01^{a}
IBM (g)	52.67 ± 0.67^{b}	59.33 ± 2.40^{a}	55.33 ± 0.67^{ab}	54.00 ± 1.15^{b}
FBM (g)	393.6 ± 42.13^{b}	477.07 ± 23.2^{b}	664.93 ± 45.52^{a}	440.29 ± 28.23^{b}
GBM (g)	340.93 ± 41.59^{b}	417.72 ± 20.87^{b}	609.69 ± 45.57^{a}	386.37 ± 27.16^{b}
Survival %	68.66 ± 3.53^{a}	45.33 ± 2.03^{d}	58.33 ± 2.91^{c}	61.67 ± 4.41^{b}

Data presented as Mean (± SE). Sample size (n) = 9 for each cross-type. All means having no lowercase superscript in common within a row are significantly different ($P< 0.05$). RR= R♀ × R♂, CC= C♀ × C♂, CR = C♀ × R♂ and RC= R♀ × C ♂.

Table 5. Early growth performance of postlarvae of all cross-types, in nursery pond under semi-intensive culture system

	Cross-types			
	Purebred		Hybrids	
	RR	**CC**	**CR**	**RC**
IBW (g)	0.26 ± 0.06^{c}	0.29 ± 0.09^{a}	0.28 ± 0.01^{ab}	0.28 ± 0.02^{bc}
FBW (g)	8.61 ± 0.30^{d}	12.87 ± 0.29^{a}	11.57 ± 0.42^{b}	10.44 ± 0.22^{c}
Wt. gain (g)	8.34 ± 0.57^{c}	12.52 ± 0.12^{a}	10.53 ± 0.49^{b}	10.93 ± 0.35^{bc}
Wt. gain% (g)	3166.1 ± 10.53^{c}	4253.57 ± 181.88^{a}	3965.52 ± 84.54^{ab}	3830.75 ± 87.43^{b}
SGR %BW/ day	3.63 ± 0.004^{c}	3.90 ± 0.05^{a}	3.87 ± 0.04^{ab}	3.79 ± 0.005^{b}
ADG (g)	0.09 ± 0.0^{c}	0.14 ± 0.0^{a}	0.12 ± 0.1^{b}	0.12 ± 0.0^{b}

Data presented as Mean (± SE). Sample size (n) = 3 for each cross-type. All means having different lowercase superscripts within a row are significantly different ($P\leq 0.05$). RR= R♀ × R♂, CC= C♀ × C♂, CR = C♀ × R♂ and RC= R♀ × C ♂.

Table 6. Growth performance indices of the fingerlings of all cross-types, after rearing in the earthen pond under semi-intensive culture system

	Cross-Types			
	Purebred		Hybrid	
Parameters	**RR**	**CC**	**CR**	**RC**
IBW (g)	8.61±0.31^{d}	12.88±0.30^{a}	11.57±0.55^{b}	10.45±0.28^{c}
FBW (g)	24.08±0.94^{c}	30.61±0.63^{a}	28.38±0.52^{b}	26.96±0.62^{b}
Wt. gain (g)	15.47±0.65^{b}	17.73±0.79^{a}	16.81±0.38^{ab}	16.51±0.39^{ab}
Wt. gain% (g)	154.73±10.67^{c}	159.45±13.63^{a}	154.35±16.29^{b}	148.87±12.11^{bc}
SGR %BW/day	2.28±0.03^{a}	2.82±0.05^{a}	2.36±0.04^{a}	2.33±0.02^{a}
ADG (g)	0.17±0.01^{b}	0.20±0.01^{a}	0.19±0.01^{ab}	0.18±0.01^{ab}

Data presented as Mean (± SE). Sample size (n) =3 for each cross-type. All means followed by similar lowercase superscript within a row are not significantly different ($P \geq 0.05$). RR= R♀ × R♂, CC= C♀ × C♂, CR = C♀ × R♂ and RC= R♀ × C ♂.

Table 7. Growth performance indices of the fingerlings of all cross-types, after 90 days rearing in the indoor facility under control conditions

	Cross-types			
	Purebred		Hybrids	
	RR	CC	CR	RC
IBW (g)	25.19±1.36^{b}	30.89±1.19^{a}	28.2±1.53^{ab}	27.29±1.89^{ab}
FBW (g)	66.95±0.45^{d}	88.14±0.88^{a}	78.66±0.56^{b}	72.76±0.76^{c}
Wt. gain (g)	42.88±1.74^{c}	55.83±1.25^{a}	49.60±0.56^{b}	45.01±0.76^{c}
Wt. gain % (g)	165.89±2.87^{c}	186.22±1.53^{a}	175.06±2.78^{b}	167.68±2.12^{c}
SGR %BW/ day	1.08±0.05^{a}	1.14±0.03^{a}	1.14±0.02^{a}	1.09±0.03^{a}
ADG (g)	0.47±0.02^{c}	0.61±0.01^{a}	0.56±0.01^{b}	0.49±0.01^{c}
IBM (g)	1889.50±16.49^{d}	2309.78±10.06^{a}	2145.00±22.18^{b}	2046.75±24.17^{c}
FBM (g)	4016.80±35.81^{d}	4848.10±108.04^{a}	4431.80±94.93^{b}	4171.84±34.56^{c}
GBM (g)	2127.31±44.51^{c}	2538.33±117.45^{a}	2286.85±72.77^{b}	2124.96±10.41^{c}
Survival %	92.30±0.58^{a}	84.61±1.15^{c}	87.77±1.92^{bc}	89.23±0.88^{b}

Data presented as Mean (± SE). Sample size (n) =3 for each cross-type. All means followed by similar lowercase superscript within a row are not significantly different ($P \geq 0.05$). RR= R♀ × R♂, CC= C♀ × C♂, CR = C♀ × R♂ and RC= R♀ × C ♂.

ADG = Average daily gain, IBM = Initial biomass, FBM = Final biomass, GBM = Gain in biomass

Table 8. Growth performance indices of all cross-types fingerlings after 90 days rearing in earthen ponds under semi-intensive culture system

	Cross-types			
	Purebred		Hybrids	
	RR	**CC**	**CR**	**RC**
IBW (g)	25.73±0.33^{c}	31.46±0.08^{a}	28.1±0.13^{ab}	26.71±0.19^{ab}
FBW (g)	222.64±3.44^{d}	407.45 ±2.97^{a}	356.79 ± 1.62^{b}	310.38 ±3.46^{c}
Wt. gain (g)	194±3.44^{d}	379.39±2.90^{a}	330±1.98^{b}	282±3.40^{c}
Wt. gain % (g)	786.01±34.27^{c}	1195.64±25.78^{a}	1172.59±45.09^{ab}	1063.60±26.34^{b}
SGR %BW/ day	2.40± 0.04^{c}	2.85± 0.02^{a}	2.82±0.03^{ab}	2.73±0.03^{b}
ADG (g)	2.19±0.02^{d}	4.18±0.04^{a}	3.65±0.03^{b}	3.15±0.03^{c}
IBM (g)	1929.5±69.97^{b}	2359.78±29.78^{a}	2107.5±67.48^{b}	2003.5±66.14^{b}
FBM (g)	16698±258.12^{d}	30558.9±222.81^{a}	26759.3±148.71^{b}	23278.5±259.5^{c}
GBM (g)	14768.5±240.1^{d}	28199.2±251.9^{a}	24651.8±191.43^{b}	21275±198.19^{c}

Data presented as Mean (± SE). Sample size (n) =3 for each cross-type. All means followed by similar lowercase superscript within a row are not significantly different ($P \geq 0.05$). RR= R♀ × R♂, CC= C♀ × C♂, CR = C♀ × R♂ and RC= R♀ × C ♂. ADG = Average daily gain, IBM = Initial biomass, FBM = Final biomass, GBM = Gain in biomass

Table 9. Fortnightly variations in the water quality parameters during the rearing of *L. rohita, C. catla* and their intergeneric hybrids in a semi-intensive culture system

Parameters	15 days	30 days	45days	60days	75 days	90 days
DO (mg/L)	5.10±0.15	5.77±0.20	6.16±0.32	5.44±0.39	6.27±0.12	5.58±0.09
pH	7.66±0.14	8.17±0.15	8.12±0.09	8.14±0.13	7.58±0.05	8.67±0.012
Temperature (°C)	26.00±0.35	27.71±0.26	28.53±0.39	29.67±0.26	29.00±0.23	28.73±0.27
TDS (mg/L)	174.36±1.81	196.73±5.31	180.00±3.31	207.57±1.56	186.57±1.86	198.79±2.23
Alkalinity (ppm)	92	167	170	173	147	140
Transparency (cm)	22	25	20	15	17	18
Ammonia (mg/L)	0.016	0.038	0.035	0.025	0.015	0.025

Table 10. Fortnightly variations in the water quality parameters during the rearing of *L. rohita, C. catla* and their intergeneric hybrids in solitary in a semi-intensive culture system

Parameters	30 days	60 days	90days	120days	150 days	180 days	230 days
Temperature (°C)	25.00±0.35	22.71±0.26	21.53±0.39	17.67±0.26	18.00±0.23	20.73±0.27	23.73±0.27
pH	7.60±0.14	7.8±0.15	7.72±0.09	7.74±0.13	7.78±0.05	7.60±0.012	7.81±0.012
DO (mg/L)	5.70±0.15	5.77±0.20	6.16±0.32	5.94±0.39	6.30±0.12	6.23±0.09	5.58±0.09
Ammonia (mg/L)	0.016	0.038	0.035	0.025	0.015	0.025	0.025

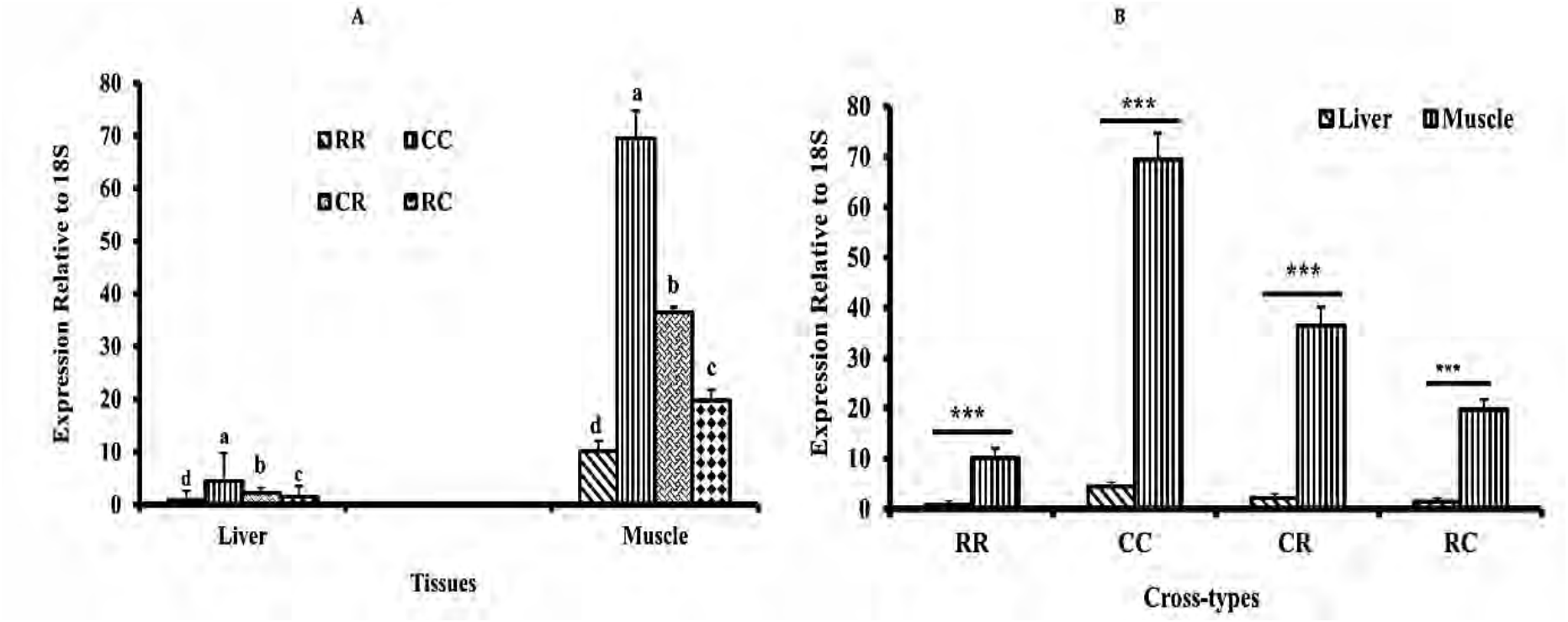


Fig. 5. Myogenin mRNA levels in muscle and liver of fingerlings of all cross-types. (A) Spatial mRNA levels among cross-types. All means having different lowercase superscripts on the bar are significantly different ($P \leq 0.05$). (B) Spatial mRNA levels within cross-type. Mean values were compared with two tail T-test. *** = $P < 0.001$. RR= R♀ × R♂, CC= C♀ × C♂, CR = C♀ × R♂ and RC= R♀ × C ♂.

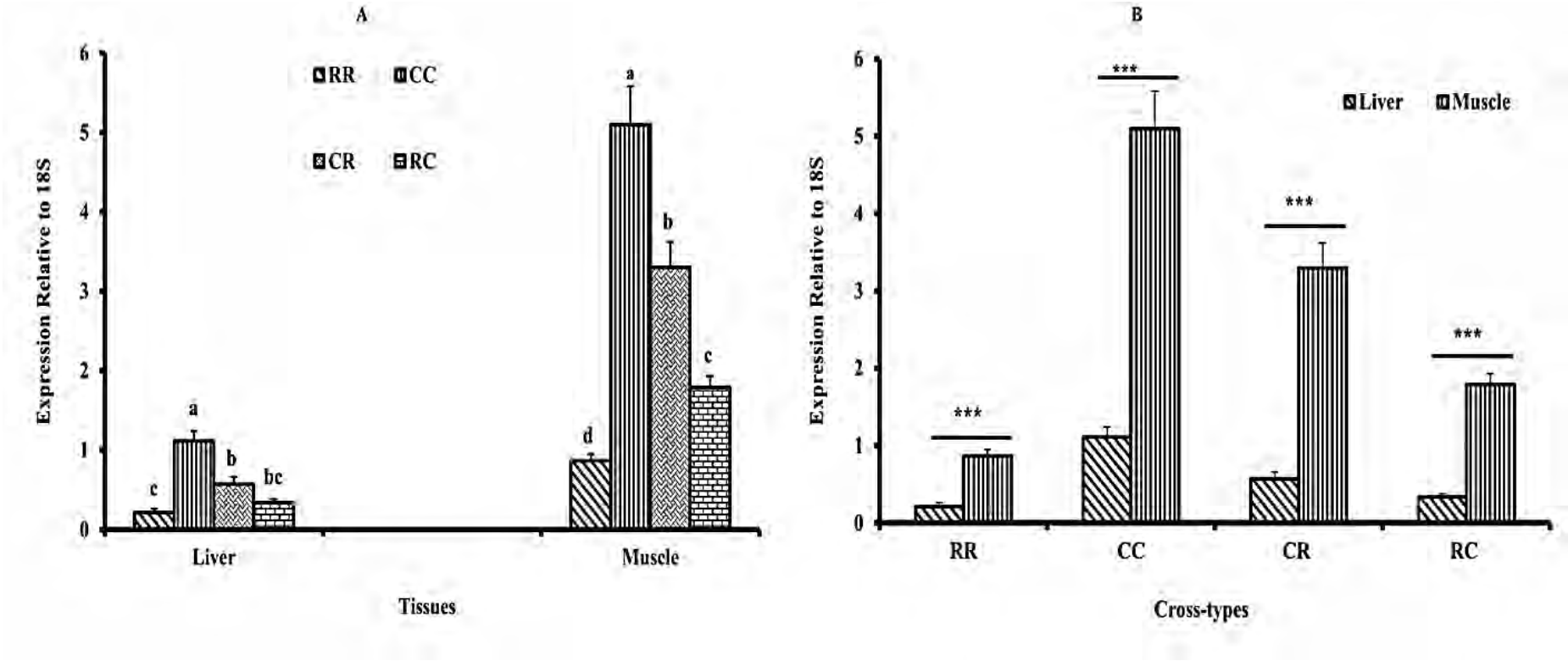


Fig. 6. MyoD mRNA levels in muscle and liver of fingerlings of all cross-types. (A) Spatial mRNA levels among cross-types. All means having different lowercase superscripts on the bar are significantly different ($P \leq 0.05$). (B) Spatial mRNA levels within cross-type. Mean values were compared with two tail T-test. *** = $P < 0.001$. RR= R♀ × R♂, CC= C♀ × C♂, CR = C♀ × R♂ and RC= R♀ × C ♂.

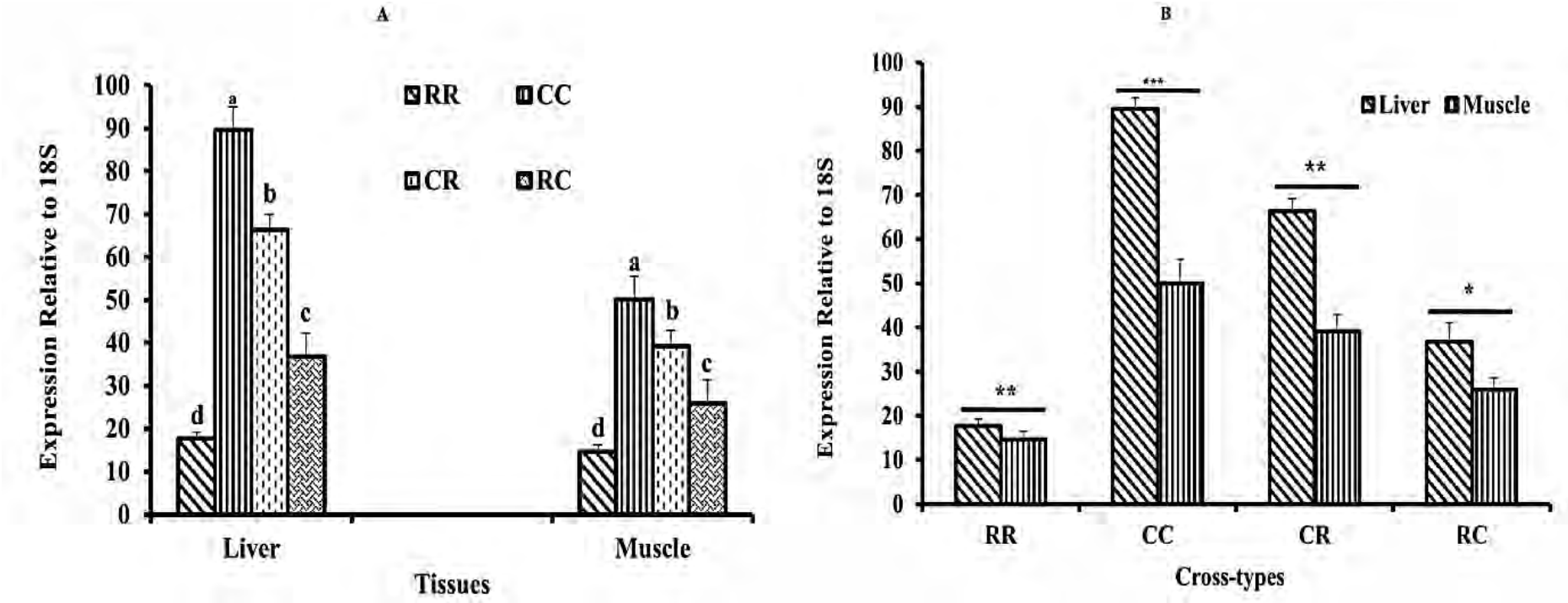


Fig. 7. IGF-1 mRNA levels in muscle and liver of fingerlings of all cross-types. (A) Spatial mRNA levels among cross-types. All means having different lowercase superscripts on the bar are significantly different ($P \leq 0.05$). (B) Spatial mRNA levels within cross-type. Mean values were compared with two tail T-test. * = $P < 0.05$; ** = $P < 0.01$;*** = $P < 0.001$. RR= R♀ × R♂, CC= C♀ × C♂, CR = C♀ × R♂ and RC= R♀ × C♂.

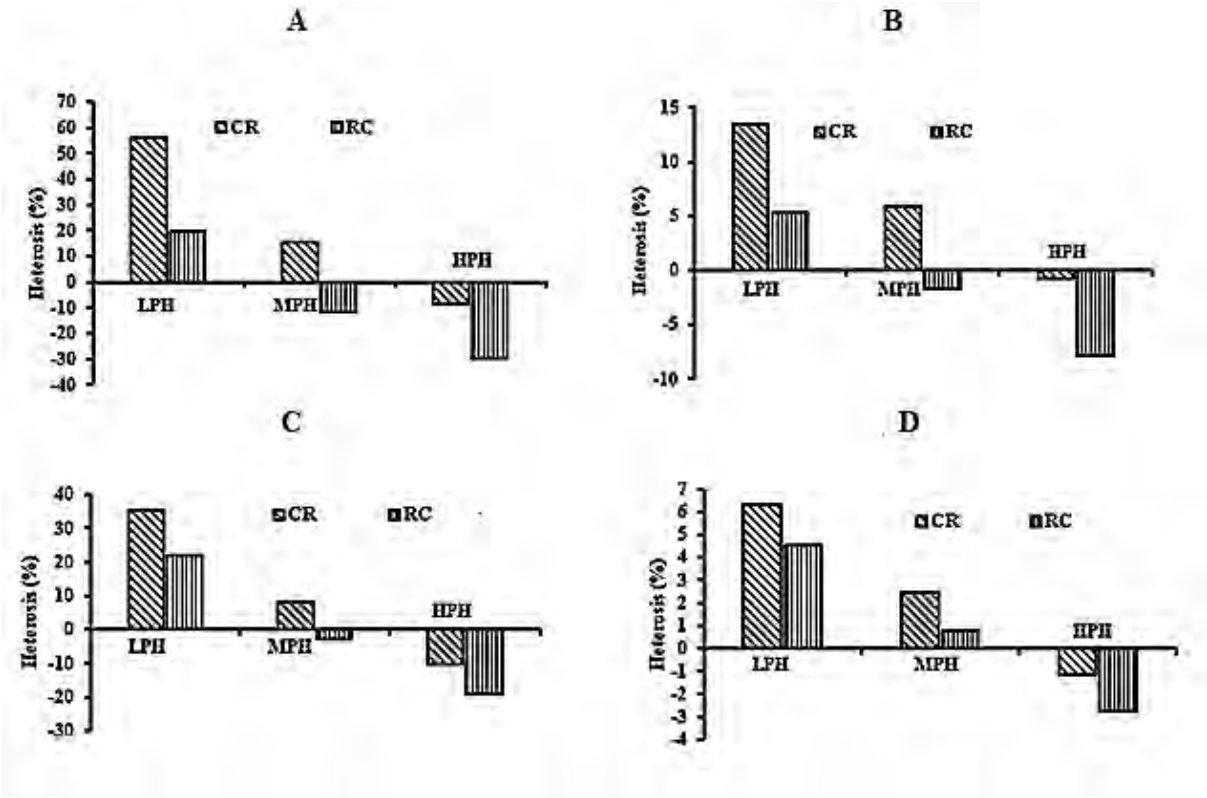


Fig. 8. Heterosis in growth related trait of CR and RC hybrids after 90 days rearing of postlarvae in controlled conditions (A) weight gain, (B) SGR%, and in semi-intensive culture system (C) weight gain, (D) SGR%

CR = C♀ × R♂ and RC= R♀ × C ♂. HPH = high parent heterosis. LPH= low parent heterosis. MPH= mid parent heterosis.

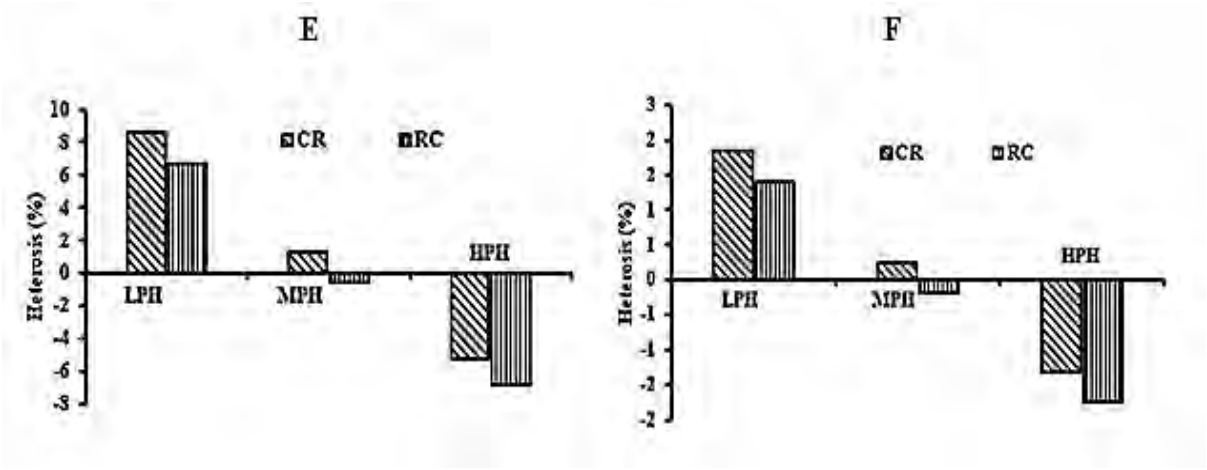


Fig. 9. Heterosis in growth related trait of CR and RC hybrids after 230 days rearing of fingerlings in the earthen pond under semi intensive culture system (E) weight gain, (F) SGR%.

CR = C♀ × R♂ and RC= R♀ × C♂. HPH = high parent heterosis. LPH= low parent heterosis. MPH= mid parent heterosis.

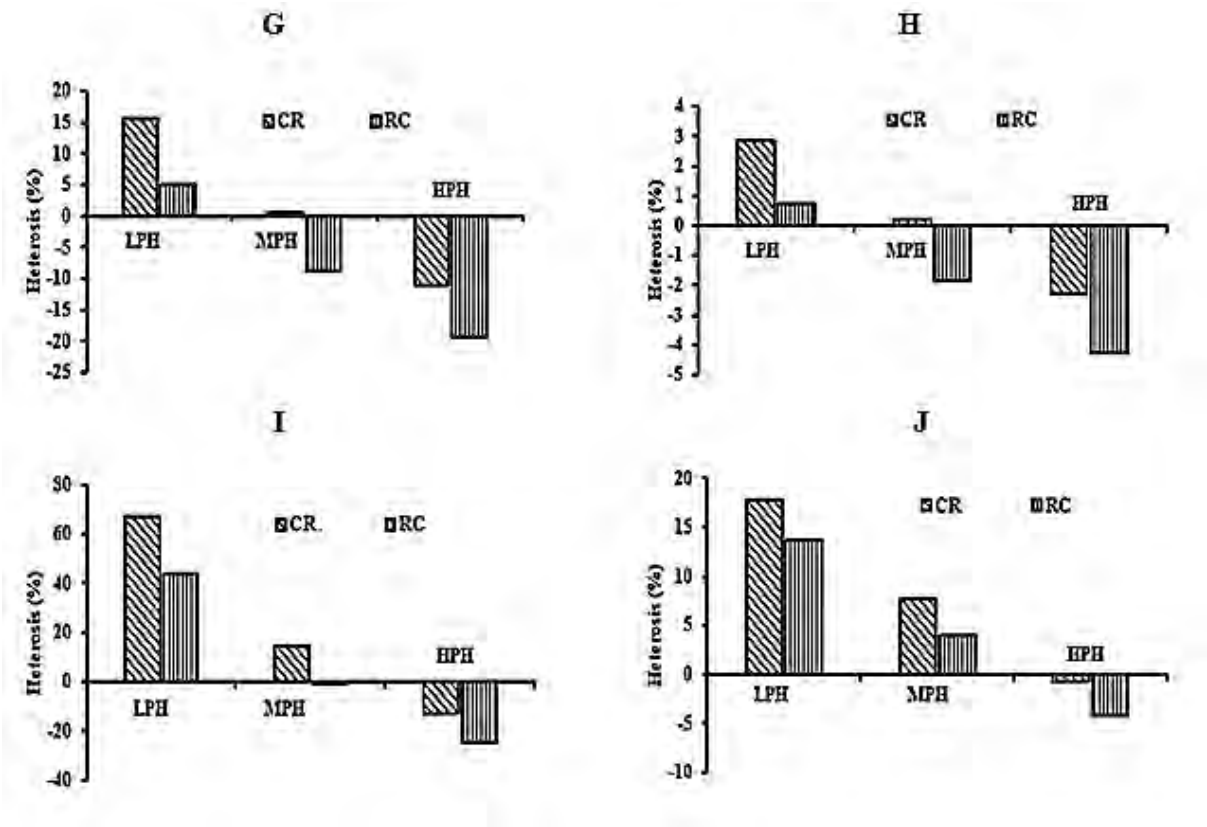


Fig. 10. Heterosis in growth related trait after 90 days rearing of fingerling in controlled conditions (G) weight gain and, (H) SGR% and the earthen ponds under semi-intensive culture system (I) weight gain and, (J) SGR %.

CR = C♀ × R♂ and RC= R♀ × C♂. HPH = high parent heterosis. LPH= low parent heterosis. MPH= mid parent heterosis.

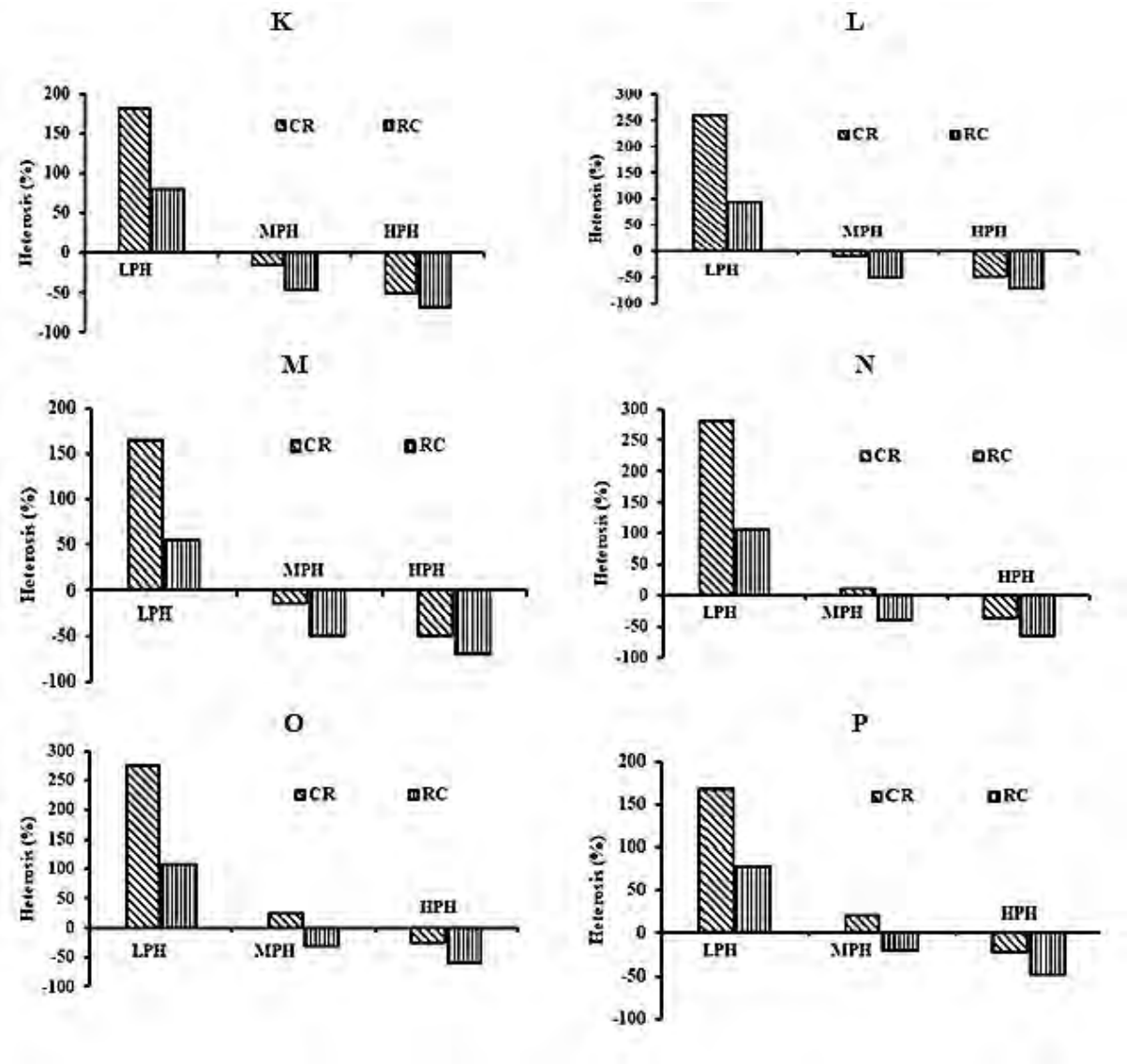


Fig. 11. Heterosis in mRNA levels of growth-related genes in CR and RC hybrids. Myogenin mRNA level in liver (K), and muscle (L), MyoD mRNA level in liver (M), and muscle (N), IGF-1 mRNA level in liver (O), and muscle (P). CR = C♀ × R♂ and RC= R♀ × C ♂. HPH = high parent heterosis. LPH= low parent heterosis. MPH= mid parent heterosis.

Chapter 2

Meristic characteristics and phylogenetic relationship of *L. rohita, C. catla* and their intergeneric hybrid produce from reciprocal crosses

Materials and Methods

For the identification of all cross-types, morphometric and meristic characteristics of fingerlings were used while molecular approach, i.e., DNA barcoding by using cytochrome c oxidase subunit I (COI) gene was used to determine the phylogenetic relationship among all cross-type.

Identification of all cross-types

The identification of the fingerlings of all cross-types was conducted by morphometric and meristic characteristics

Morphometric approach

For the morphometric study, Kamboj & Kamboj (2019) approach was adopted and Total and Standard length (cm), Head length and depth (cm), Snout length, Forked length (cm), Eye diameter (cm), Pre- and post dorsal length (cm), Pre- and post pectoral length (cm), Pre- and post pelvic length (cm), Pre- and post anal fin length (cm), Body girth and depth (cm) of the fingerlings of all cross-types were recorded and some selected ratios were also calculated.

Meristic approach

For meristic traits analysis, fin rays of pectoral (P), dorsal (D), caudal (C), ventral (V), anal (A) fins, transverse scale and scales on the lateral line were counted.

Statistical Analysis

All morphometric and meristic traits are presented as Mean (± SE). By using statistical software (Statistic version 8.1 and SPSS, version 20), One-way analysis of variance (ANOVA) and LDS tests were performed to determine any potential significant difference among the means of morphometric and meristic traits of all cross-types.

Phylogenetic relationship

Sample collection and preservation

Fingerlings of all cross-types (Parent species (RR and CC as well as their reciprocal hybrids, CR and RC) were collected, anaesthetized with buffered MS222 and the pectoral fin of each fish was clipped. These fins of each cross-type were washed thoroughly in 75% alcohol and suspended in ethanol absolute (95 %) and stored at low temperature (-20˚ C) until further processing.

DNA extraction and amplification

A modified salt extraction method was used to extract the DNA**.** Amplification of the 670bp barcode region of the mitochondrial DNA was performed using already reported primers (Ward et al., 2005). Primers were synthesized from MACROGEN Inc., Seoul, Korea.

Table 11. Primer of COI

Primer	Primer sequence (5′- 3′)
Fish F1	TCAACCAACCACAAAGACATTGGCAC
Fish R1	TAGACTTCTGGGTGGCCAAAGAATCA

PCR reaction was carried out by gradient thermal cycler PCR (BIO-RAD T100™ thermal cycler) by using 30µL mixture (the composition mentioned in Table 12) and conducting PCR under conditions mentioned in Table. The final product was stored at 4^{o}C. To make sure that the PCR reaction yield adequate amplicon sizes, gel electrophoresis of PCR products was performed and visualized the bands on 1.5 % agarose gel containing ethidium bromide. Samples showing the good quality of bands were selected and sent for sequencing to MACROGEN, (Korea) using forward and reverse primers.

Table 12. Reaction mixture for Polymerase chain reaction

Reagents	**Volume (µl)**
DNA	3 µg/µl
Forward primer	1
Reverse primer	1
PCR Master mix	14
PCR water	11
Total reaction mixture	30

Table 13. PCR reaction conditions

Steps	Temperature °C	Time	Cycle
Initial denaturation	94	4mint	1
Denaturation	94	60sec	35
Annealing	54	45 sec	
Extension	72	60 sec	
Final Extension	72		1
Final storage	-4	Infinity	

Bioinformatics Analysis

The obtained sequence was edited and aligned by using Bio edit software and ClustalW respectively (Thompson et al., 1997). The range of difference in sequence among different cross-types was determined by the K2P model (Kimura 2-parameter) (Kumar et al., 2018). For phylogenetic analysis, the neighbour-joining and Maximum likelihood cladogram was constructed by using Molecular Evolutionary Genetics Analysis version 7 (MEGA Inc., Englewood, NJ)

Results

Phenotypic traits

Phenotypic traits like head shape and size, eye diameter, fin color, lateral line, scale shape, body shape and colour of the fish of all cross-types are shown in (Table 14). The external morphological features indicated that both hybrids (RC and CR hybrids) were intermediate in most of the traits, while some traits resemble their mother species. For instance, *L. rohita* had a terminal mouth with fringed lips whereas *C. catla* showed upturned month with infringed lips. However, the mouth gap and lateral line of both hybrids were similar to the mother.

Purebred species

RR cross-type

RR cross-type, i.e., *L. rohita*, the body shape was fusiform. The mouth was sub-terminal with square or somewhat circular mouth gap/opening. Position of eyes was dorso-lateral. Lower lip was folded and fringed while upper lip extending and covered the lower lip. Pelvic fins were red while the lateral line was slightly curved. Except forthe head and fins, the whole body was covered with cycloid scale. The exposed portion of the scale was a diamond shape. Dorsal side of the body was blackish while silvery at the sides and below.

CC cross-type

CC cross-type, i.e., *C. catla* body was deep and short, fairly laterally compressed with a conspicuously broad and large head. Larger cycloid scales cover the whole body except head. The mouth was wide, upturned with circular large opening/gap and protruding lower jaw. The eyes were large while lower part of the eyeball had red spot, that visible from the underside of the head. The upper lip was thin while the lower lip was infringed and thick/folded. All fins were darkish in color. The lateral line was curved. The dorsal side of the body was greyish while the ventral region was silvery white.

Hybrids

CR cross-type

In morphometric features, CR hybrid resembled *C. catla* in body appearance and *L. rohita* in mouth profile. The body was relatively deep, dorsal profile far more convex as compared to ventral profile. The mouth was terminal, moderately wide with circular opening and a slightly fringed lower lip. The body was slightly greenish on the dorsal side. Like *C. catla* , pectoral, ventral and anal fins were black.

RC cross-type

The body depth of RC is wider than *RR* but not like CC. The head size was somewhat larger than RR but smaller than CC. The mouth was terminal with an intermediate size circular opening. The upper lip was extended and covered the lower lip while the lower lip was folded and not fringed. Pelvic fins are red-colored while other fins are darkish in color. Lateral line curved as in *C. catla*. Like RR, the exposed portion of scales was diamond shape. Moreover, like CC dorsal region color was greyish while like RR , ventral region was white

(a) Rohu (b) Catla (c) CR hybrid (d) RC hybrid

Morphometric measurements and morphometric ratio

Morphometric measurements like standard and total length (SL and TL respectively), fork length (FL), head length (HL), snout length (SnL), predorsal length (PreDL), post dorsal length (PDL), post pectoral length (PPL), post pelvic length (PPL), body depth and body girth of the fish of all cross-types are presented in (Table 14), while certain morphometric ratios are given in (Table 15). Statistical analysis of variance followed by post hoc LSD test indicated that total length ratio with SL, FL, HL, SnL, PreDL, PDL, PPL, PPD, BD and BG among all cross-type were statistically similar ($P<0.05$), while TL ratio with HD, ED, PrePL and PreAL were significantly different ($P>0.05$) among cross-type. The CC cross-type (*C. catla*) showed higher values of TL ratio with HD, ED and PAL and lower values with PrePL and PreAL as compared to other cross-types.

Meristic characters

Eighteen countable characters, i.e., meristic characters of all cross-types were counted including scales below and above lateral line, numbers of dorsal fin rays, and numbers of caudal fin rays, numbers of anal fin rays, numbers of pectoral fin rays and numbers of pelvic fin rays of the fish of all cross-types. We did not find any substantial difference ($P<0.05$) among fish of all cross-types to the scale on lateral line, number of un-branched dorsal fin rays, the total number of pelvic fin rays, branched pelvic-fin rays, the total number of caudal fin rays, anal fin rays, branched and unbranched anal-fin rays. However, the scale below and above lateral line, numbers of dorsal fin rays, pectoral fin rays, branched pectoral-fin rays, unbranched pectoral-fin rays, unbranched pelvic fin rays, branched caudal-fin rays, and number of unbranched caudal fin ray showed a significant difference ($P<0.05$) among fish of all cross-types (Table 17)

Table 14. Phenotypic traits of all cross-types (RR, CC, CR, RC)

	Cross-types			
	Purebred		Hybrid	
	RR	**CC**	**CR**	**RC**
Head size	Arched Smaller	Large broad head	Smaller than *C. catla* but somewhat larger than *L. rohita*.	smaller than *C. catla* but somewhat larger than *L. rohita*.
Body shape	Fusiform	Short, deep, somewhat laterally compressed	relatively deep, compressed with the dorsal profile far more convex as compared to ventral profile	Body depth is wider than rohu but not so as Catla
Mouth	Sub-terminal	Upturned	Terminal	Terminal
Mouth gap	Square	Circular	Circular	Circular
Lower lip	Fringed and folded	Thick/folded and infringed	Lower lip slightly fringed	Folded and not fringed
Eye	No red spot on the lower part of eyeball	On the lower part of eye ball red spot	No red spot on lower part of eyeball	No red spot on lower part of eyeball
Pelvic fins	Red in color	Darkish in color	Red colored Black	Red colored
Lateral line.	Slightly curved	curved	curved	Slightly curved
Scale	Cycloid, exposed portion diamond shape	Larger cycloid scales	Cycloid scale. intermediate size Diamond shaped	Cycloid scale. intermediate size

RR= R♀ × R♂, CC= C♀ × C♂, CR = C♀ × R♂ and RC= R♀ × C ♂.

Table 15. The average values of various morphometric parameters of all cross-types

	Cross-types			
	Purebred species		Hybrids	
	RR	CC	CR	RC
Total Length (TL)	12.3±0.66	13.54±1.47	10.84±0.47	9.88±0.50
Standard Length (SL)	9.72±0.58	11.04±1.25	8.54±0.37	7.82±0.43
Fork Length (FL)	10.56±0.64	11.98±1.36	9.42±0.37	8.62±0.45
Head Length (HL)	2.1±0.10	2.44±0.26	2.06±0.05	1.8±0.05
Head Depth (HD)	1.5±0.10	1.74±0.17	1.5±0.07	1.08±0.04
Snout Length (SnL)	1.34±0.08	1.66±0.17	1.38±0.16	1.12±0.06
Eye Diameter (ED)	0.7±0.01	0.76±0.04	0.66±0.07	0.44±0.04
Pre Dorsal Length (PDL)	4.48±0.21	5.88±0.16	3.92±0.15	4±0.19
Post Dorsal Length (PDD)	7.86±0.47	7.62±0.72	6.88±0.32	6.04±0.43
Pre Pelvic Length (PPL)	2.44±0.17	3.1±1.33	3.52±1.15	2.2±0.19
Post Pelvic Depth (PPD)	9.84±0.55	10.38±1.10	7.34±1.32	7.84±0.43
Pre Pelvic Length (PPL)	4.96±0.24	5.075±0.62	4.34±0.14	4.3±0.27
Post Pelvic Length (PPD)	7.34±0.42	8.02±0.85	6.4±0.34	5.72±0.36
Pre Anal Length (PAL)	7.52±0.40	8.18±0.91	6.24±0.24	7.4±0.18
Post Anal Depth (PAD)	4.78±0.26	5.33±0.18	4.12±0.24	2.64±0.04
Body Depth (BD)	2.54±0.16	3.95±0.52	3.08±0.14	2.3±0.23
Body Girth (BG)	5.46±0.33	8.92±0.57	5.84±0.18	5.38±0.47

RR= R♀ × R♂, CC= C♀ × C♂, CR = C♀ × R♂ and RC= R♀ × C ♂.

Table 16. Body proportions of taxonomic characteristic of all cross-types

		Cross-types				
		Purebred species		Hybrids		
		RR	CC	CR	RC	P-value
Standard Length	TL/SL	1.27±0.01[a]	1.23±0.03[a]	1.27±0.01[a]	1.26±0.01[a]	0.18
Fork Length	TL/FL	1.17±0.01[a]	1.14±0.02[a]	1.15±0.01[a]	1.15±0.01[a]	0.53
Head Length	TL/HL	5.85±0.11[a]	5.54±0.12[a]	5.29±0.35[a]	5.48±0.12[a]	1.36
Head Depth	TL/HD	8.25±0.31[b]	7.77±0.21[bc]	7.27±0.35[c]	9.13±0.18[a]	0.00
Snout Length	TL/SnL	9.21±0.24[a]	8.13±0.08[a]	8.41±1.28[a]	8.83±0.09[a]	0.68
Eye Diameter	TL/ED	17.57±0.94[b]	17.66±1.33[b]	16.57±1.19[b]	22.77±1.07[a]	0.01
	HL/Snl	1.57±0.02[a]	1.47±0.04[a]	1.56±0.16[a]	1.61±0.04[a]	0.69
	Snl/ED	1.91±0.12[b]	2.17±0.14[b]	2.07±0.17[ab]	2.58±0.14[a]	0.03
	HL/ ED	3.00±0.14[b]	3.19±0.25[b]	3.13±0.06[b]	4.17±0.22[a]	0.00
Pre-dorsal length	TL/PreDL	2.74±0.03[a]	2.49±0.22[a]	2.77±0.07[a]	2.47±0.01[a]	0.18
Post-dorsal Length	TL / PDL	1.57±0.02[a]	1.81±0.23[a]	1.58±0.02[a]	1.64±0.03[a]	0.44
Pre-pectoral length	TL/PrePPL	5.08±0.23[a]	4.38±0.11[a]	3.97±0.72[b]	4.54±0.15[a]	0.28
Post-pectoral length	TL / PPL	1.25±0.01[a]	1.30±0.01[a]	1.89±0.61[a]	1.26±0.01[a]	0.40
Pre-pelvic length	TL/PrePL	2.48±0.02[a]	2.48±0.04[a]	2.50±0.04[a]	2.30±0.03[b]	0.00
Post-pelvic length	TL / PPL	1.68±0.01[a]	1.69±0.02[a]	1.70±0.02[a]	1.73±0.03[a]	0.30
Pre-anal length	TL/PreAL	1.64±0.01[a]	1.66±0.02[a]	1.79±0.19[a]	1.33±0.03[b]	0.00
Post-Anal length	TL/PAL	2.57±0.01[b]	2.57±0.07[b]	2.50±0.09[b]	3.99±0.38[a]	
Body depth	TL/BD	4.86±0.04[a]	3.64±0.15[a]	4.60±0.28[a]	4.38±0.23[a]	0.95
Body Girth	TL/BG	2.26±0.08[a]	1.58±0.06[a]	1.86±0.11[a]	1.86±0.07[a]	0.70

Data presented as Mean (± SE). All means having no lowercase superscript in common within a row are significantly different from each other (P< 0.05). RR= R♀ × R♂, CC= C♀ × C♂, CR = C♀ × R♂ and RC= R♀ × C ♂.

Table 17. Meristic characters of all cross-types

	Cross-type				
	Purebred species		Hybrids		
	RR	CC	CR	RC	P value
Scales on lateral line	41.2±0.37^{a}	42.2±0.37^{a}	42.6±0.23^{a}	41.6±0.60^{a}	0.13
Scale above lateral line	8.4±0.24^{b}	8.6±0.24^{b}	9.4±0.23^{a}	10.00±0.45^{a}	0.01
Scales below lateral line	6.6±0.24^{b}	6.8±0.37^{b}	10.6±0.22^{a}	9.4±0.68^{a}	0.00
Total No of dorsal fin rays	15.2±0.37^{b}	17.4±0.24^{a}	16.6±0.24^{a}	16.2±0.37^{ab}	0.00
No of branched dorsal fin rays	12.6±0.24^{b}	14.5±0.22^{a}	13.6±0.24^{ab}	14.2±0.37^{a}	0.00
Nor of un-branched dorsal fin rays	2.4±0.24^{a}	2.5±0.22^{a}	2.8±0.58^{a}	2.00±0.0^{a}	0.19
Total No of pectoral fin rays	14.2±0.37^{c}	15.8±0.49^{bc}	18.4±0.24^{a}	17.2±0.37^{ab}	0.00
No of branched pectoral fin rays	12.4±0.24^{b}	14.3±0.23^{a}	15.0±0.45^{a}	14.6±0.40^{a}	0.00
No. of un-branched pectoral fin rays	1.4±0.24^{b}	1.4±0.24^{b}	3.40±0.68^{a}	2.6±0.24^{ab}	0.01
Total No. of pelvic fin rays	9.0±0.00^{a}	8.8±0.24^{a}	9.4±0.24^{a}	9.4±0.24^{a}	0.12
No. of branched pelvic fin rays	7.5±0.22^{a}	7.7±0.24^{a}	7.4±0.22^{a}	7.6±0.23^{a}	0.70
No. of un-branched pelvic fin rays	1.4±0.24^{ab}	1.0±0.00^{b}	2.00±0.00^{a}	1.6±0.20^{ab}	0.00
Total No. of caudal fin rays	18.4±0.24^{a}	19.0±0.00^{a}	18.8±0.37^{a}	19.4±0.24^{a}	0.08
No. of branched caudal fin rays	14.2±0.24^{c}	17.0±0.00^{a}	15.6±0.24^{b}	14.8±0.37^{bc}	0.00
No. of un-branched caudal fin rays	5.0±0.58^{a}	2.0±0.00^{c}	3.6±0.24^{ab}	3.2±0.37^{bc}	0.00
Total No. of anal fin rays	7.4±0.24^{a}	8.0±0.45^{a}	8.00±0.00^{a}	7.6±0.24^{a}	0.37
No. of branched anal fin rays	6.0±0.00^{a}	6.0±0.00^{a}	5.6±0.24^{a}	5.6±0.24^{a}	0.19
No. of un-branched anal fin rays	1.4±0.24^{a}	2.0±0.45^{a}	2.4±0.24^{a}	2.00±0.45^{a}	0.31

Data presented as Mean (± SE). All means having no lowercase superscript in common within a row are significantly different from each other ($P< 0.05$). RR= R♀ × R♂, CC= C♀ × C♂, CR = C♀ × R♂ and RC= R♀ × C ♂.

Phylogenetic Relationship

In the present study, a molecular approach i.e., DNA barcoding by using mitochondrial COX 1 gene was undertaken to differentiate the fish of all cross-types (RR, CC, RC, and CR). The constructed Neighbour Joining (NJ) tree (K2P) based on barcode sequencing with a region of approximately 680bp for fish of four cross-types i.e., (RR, CC, CR and RC hybrids), is shown in (Fig 14). NJ tree revealed two clades, which indicated the two separate species. The analyses of the histogram of genetic and ranked distances revealed a clear gap between conspecific and congeneric species. Each hybrid was closely related to its female parent and showed an identical phylogenetic relationship with the mother species. For instance, CR hybrids showed the minimum distance with *C. catla* and RC hybrid showed the minimum distance with *L. rohita*. Based on K2P model, the interspecific divergence between parent species and hybrid range from 0.003-0.014 (Table 19). The maximum genetic distance was noticed between *C. catla* and RC hybrid and the lowest distance was observed between *L. rohita* and RC hybrid.

Organization and composition of mitochondrial Cox-1 region

COI gene sequence analyses of RR cross-type

CO1 sequence analysis of 14 individuals of RR cross-type (*L. rohita*) indicated the length of the average nucleotide of 670bp excluding sites with alignment gaps or missing data. Among these, 656 sites (97.9%) were invariable (monomorphic), while 14 sites (2.1%) were variable (polymorphic). Among polymorphic sites, 9 at 17, 64, 106, 130, 148, 149, 234, 318, and 577 positions were singleton while 5 at 268, 338, 415, 490, and 628 site positions were parsimony informative. In RR cross-type, a total number of 4 haplotypes with a diversity of0.455 haplotype (HD) and nucleotide diversity (Pi), 0.029 were observed. The mean inter-specific K2P genetic distance was 0.003. Sequence analysis revealed the composition of nucleotide of CO1 gene in *Labeo rohita* as G, 17.9%;A, 26.8%; C, 27%;T, 28.3%; with integrated composition as, A+T= 55.1%, G+C= 44.9%.Furthermore, the estimated Transition/transversion bias (R) value was 0.25, while the rate of transitional and transversional substitution was 10.11% and 4.98% respectively. Tajima D, Fu and Li, D and F tests were also applied and found negative values (Tajima D: -1.49161; Fu and Li's D test: -1.16700; Fu and Li's F test: -1.29477).

COI gene sequence analyses of CC cross-type

CO1 sequence analysis of 17 individuals of CC cross-type (*C. catla*) indicated the mean nucleotide length of 672bp excluding the sites with alignment gaps or missing data. Among these, 655 sites (97.5%) were invariable (monomorphic), while 17 sites (2.5%) were variable (polymorphic). Among polymorphic sites, 11 at 116, 393, 416, 491, 502, 522, 577, 579, 597, 633 and 663 positions were singleton while 6 sites at 65, 218 235, 314, 431 and 623 were parsimony informative. A total number of 5 haplotypes, with haplotype diversity (Hd) 0.476 and nucleotide diversity (Pi), 0.024 were observed in CC cross-type. The mean intraspecific K2P genetic distance was 0.002. Sequence analysis revealed the nucleotide composition of CO1 gene in *C. catla as* T, 28.2%; C, 27.1%; A, 26.7%; G, 18% with integrated composition as, A+T= 54.9%, G+C= 45.1%. Furthermore, the estimated Transition/transversion bias (R) value was 0.95, while the rate of transitional and transversional substitution was 12.53% and 6.23% respectively. Tajima D, Fu and Li, D and F tests were also applied and found negative values (Tajima D: -1.7591; Fu and Li's D test: -1.475; Fu and Li's F test: -1.785).

COI sequence analysis of CR cross-type

CO1 sequence analysis of 16 individuals of CR cross-type (hybrid) indicated an average read length of 644 bp excluding the sites with alignment gaps or missing data. The 644bp consisted of 633 (98.2%) invariable (monomorphic) and 11 (1.71%) variable polymorphic sites. The variable sites at 6 positions, i.e., 108, 112, 119, 212, 593 and 602 were singleton, while at other 5 site positions, 590, 592, 608, 641 and 642 were parsimony informative. In CR cross-type, a total number of 6 haplotypes, with Hd = 0.844 and Pi = 0.005 were observed. The mean intraspecific K2P genetic distance was 0.005. The nucleotide composition of CO1 region in CR hybrid was T, 29.2%; C, 24.1%; A, 26.9%; G, 19.8% with 56.1% A+T and 43.9% G+C contents. Furthermore, the estimated Transition/transversion bias (R) value was 1.20, while the rate of transitional and transversional substitution was 13.64% and 5.68% respectively. Tajima D, Fu and Li, D and F tests were also conducted and found all negative values (Tajima D: -0.79269; Fu and Li's D test: -0.57230; Fu and Li's F test: -0.70523).

COI sequence analysis of RC cross-type

CO1 sequence analysis of 13 individuals of RC cross-type (hybrid) indicated the mean nucleotide length of 659 bp excluding the sites with alignment gaps or missing data. Among these, 650 sites (98.64%) were invariable (monomorphic), while only 9 sites (1.37%) were variable (polymorphic). The variable sites at 203, 228, 512, 576, 626, 655 and 658 position were singleton, while only at 2 positions, i.e., at 220, 254 sites were parsimony informative. In RC hybrid, a total number of 4 haplotypes with Hd = 0.583 and Pi= 0.0042 were observed. The mean intraspecific K2P genetic distance was found to be 0.003. The nucleotide composition of CO1 region in RC hybrid was T= 28.3%; C=24.9%; A = 26.1%; G= 20.7% with 54.4% A+T and 45.6% G+C contents. The estimated Transition/transversion bias R-value was 1.05. The rate of transitional substitution was 11.32%, while transversional substitution was 4.72%. To detect sequence variability Tajima D, Fu and Li, D and F tests were conducted and found all values <0 (Tajima D test = -1.55, Fu and Li's D test = -1.02 and Li's F test: -1.08)

Similarities and variations in sequence

Comparison of the sequence of Cox1 of mitogenomes among the female parents species and their hybrids revealed sequence similarities above 97 percent in all cases.*C. catla* and *L. rohit* has 0.011evalutionary divergence, while the divergence between both hybrids, CR cross-type and RC cross-type was 0.009, but the divergence between *L. rohita* and CR hybrid was 0.006. Similarly, the divergence between *C. catla* and CR Hybrid found 0.006, a divergence between *L. rohita* and RC Hybrid was 0.003, while *C. catla* and RC Hybrid was 0.014,

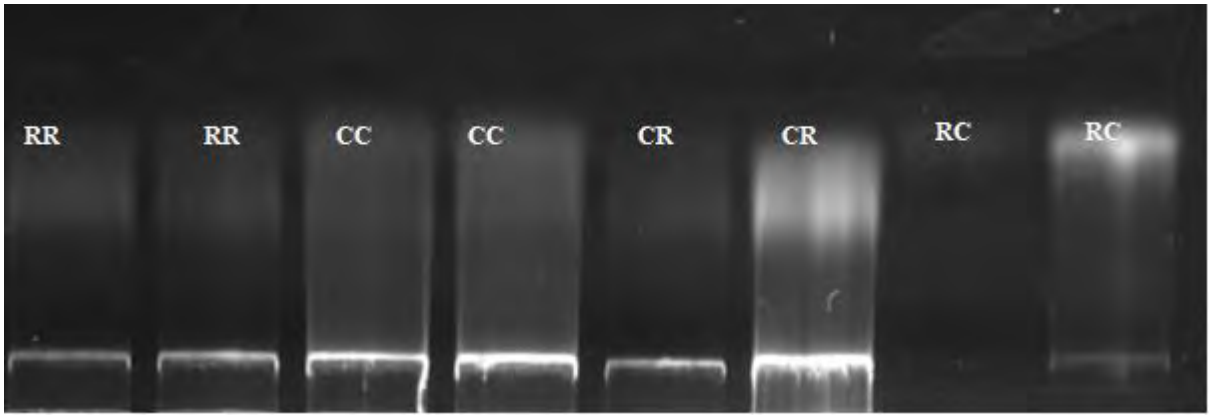


Fig. 12. Agarose gel DNA bands of *L. rohita C. catla* and their intergeneric hybrid produced from reciprocal crosses. RR= R♀ × R♂, CC= C♀ × C♂, CR = C♀ × R♂ and RC= R♀ × C♂.

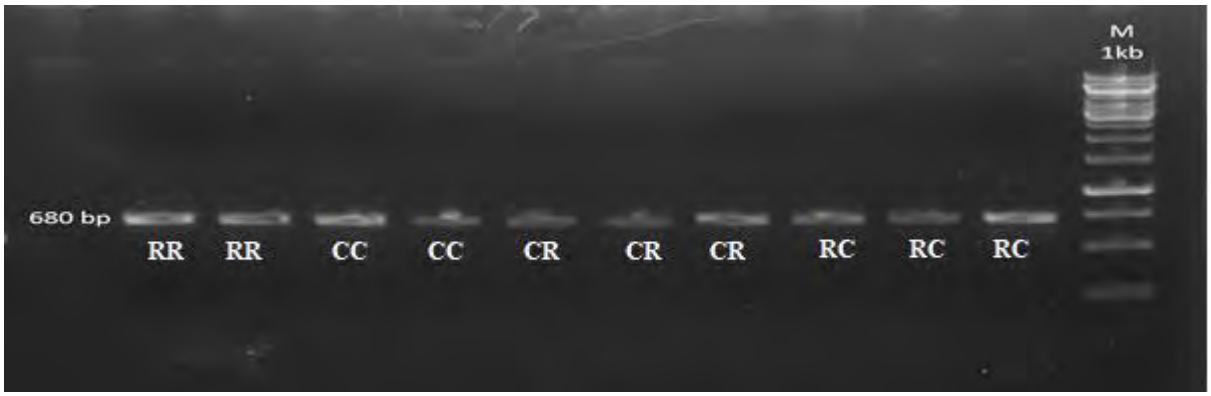


Fig.13. Gel electrophoresis image of amplified PCR product of CO I gene of all cross-types. RR= R♀ × R♂, CC= C♀ × C♂, CR = C♀ × R♂ and RC= R♀ × C ♂.

Table 18. CO1 gene sequence accession number of all cross-types

	Cross-types			
	Purebred		Hybrid	
	RR	**CC**	**CR**	**RC**
1	MN964134	MN964128	MT954411	MN964131
2	MN964135	MN964129	MT954412	MN964132
3	MN964136	MN964130	MT954413	MN964133

RR= R♀ × R♂; CC= C♀ × C♂; CR = C♀ × R♂ and RC= R♀ × C ♂.

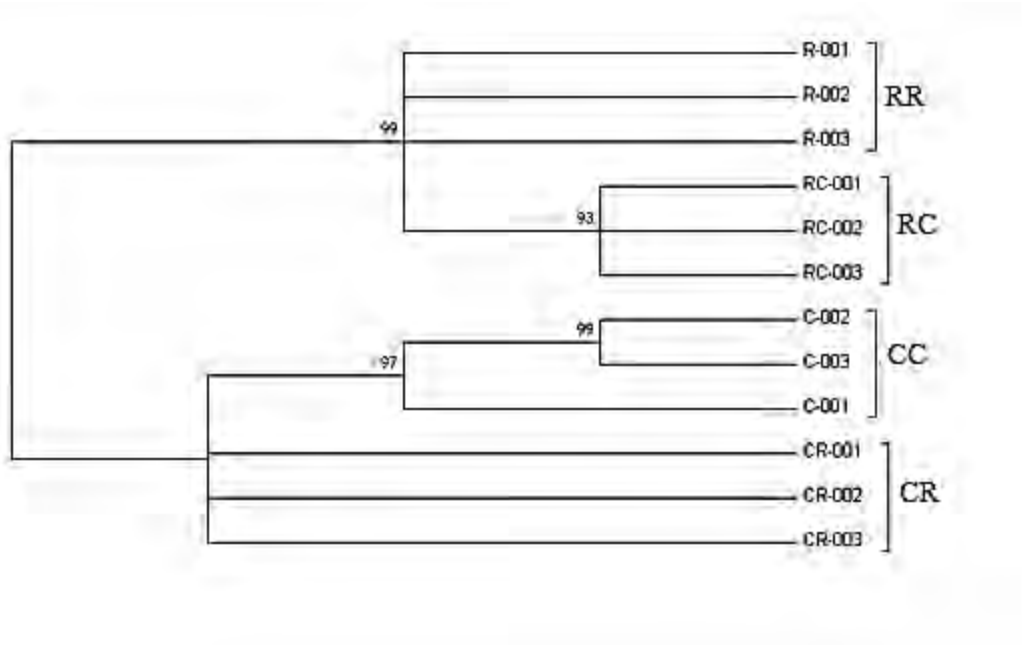


Fig. 14. NJ-Phylogenetic tree (K2P) tree showing relationship among four cross-types RR= R♀ × R♂; CC= C♀ × C♂; CR = C♀ × R♂ and RC= R♀ × C ♂.

Table 19. Estimate evolutionary divergence over sequence pairs among all cross-types (RR, CC, CR, RC)

	Cross-types			
	Purebred		Hybrid	
	CC	**CR**	**RC**	**RR**
CC				
CR	0.005			
RC	0.014	0.009		
RR	0.011	0.006	0.003	

RR= *L. rohita*♀×*L. rohita*♂ CC= *C. catla*♀× *C. catla*♂, CR = *C. catla*♀×*L. rohita*♂ RC= *L. rohita*♀×*C. catla*♂

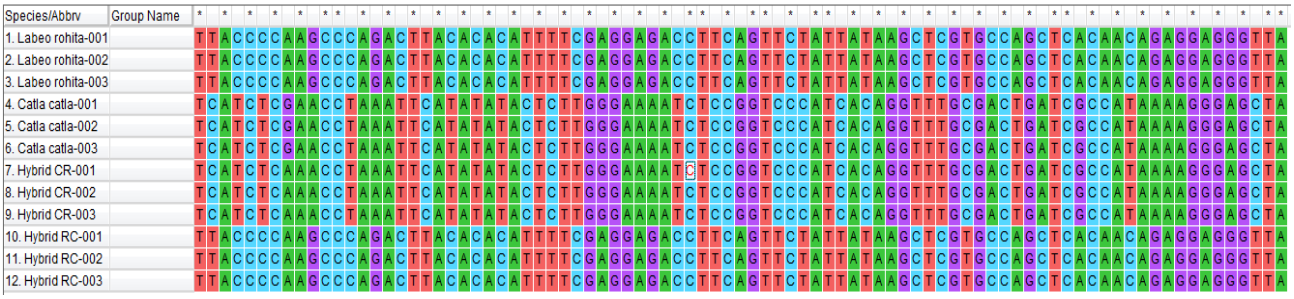


Fig. 15. Mutational analysis of four cross-types

RR= R♀ × R♂; CC= C♀ × C♂; CR = C♀ × R♂ and RC= R♀ × C ♂.

Table 20. Nucleotide variation among all cross-types

Nucleotide (%)	Cross-types			
	Purebred		Hybrid	
	RR	CC	CR	RC
T	28.3	28.2	29.2	28.3
C	27	27.1	24.1	24.9
A	26.8	26.7	26.9	26.1
G	17.9	18	19.8	20.7
A+T	55.1	54.9	56.1	54.4
G+C	44.9	45.1	43.9	45.6

RR= R♀ × R♂; CC= C♀ × C♂; CR = C♀ × R♂ and RC= R♀ × C ♂.

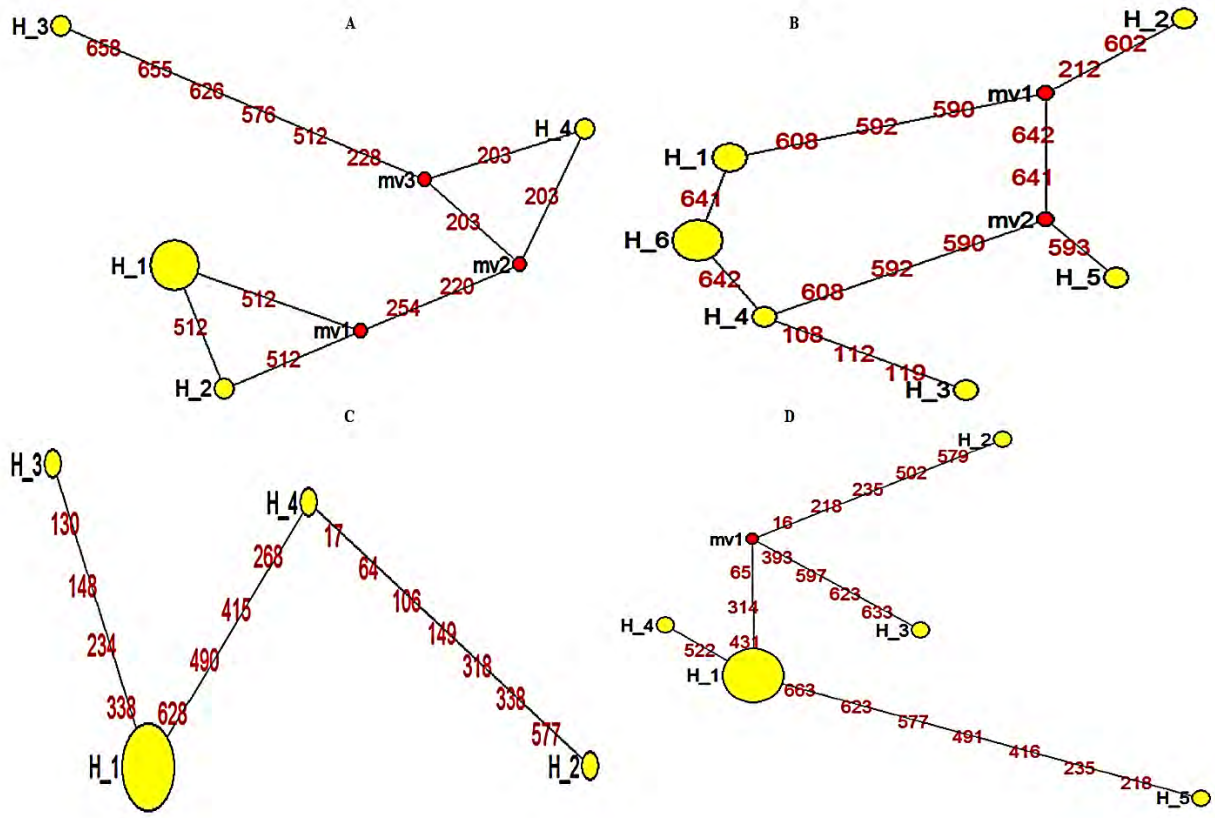


Fig. 16. Haplotypes along with variation sites of *L. rohita C. catla* and their intergeneric hybrid produced from reciprocal crosses (a) RR= R♀ × R♂ (b) CC= C♀ × C♂; (c) CR = C♀ × R♂ (d) RC= R♀ × C ♂.

Chapter 3

Comparative disease resistance and immune response of *Catla catla*, *Labeo rohita* and intergeneric hybrids after exposure to *Aeromonas hydrophila*

Materials and Methods

The research was conducted in collaboration with Tawakkal Fish Hatchery & Farm, Muzaffargarh. The induce breeding of two closely and phylogenetically related species Rohu (*L. rohita*) and Thaila (*C. catla*) was conducted and parental species as well intergeneric hybrid were produced at Tawakkal hatchery while comparative immunity and disease resistance tests were performed at Fisheries and Aquaculture facility, Quaid-i-Azam University.

Fish breeding and production of hybrids

Progeny of *L. rohita* and *C. catla* (Tawakal hatchery strains) which were induced bred on the same day with induced spawning agent ovaprim (0.2 and 0.5 ml/kg of male and female respectively) was used for this study. Before stripping of female broodfish, milt of all *C. catla*as well as *L. rohita* was collected separately in test tubes and diluted with 0.9% NaCl. For producing parental species and reciprocal intergeneric hybrids, the pooled milt samples were used to fertilize the eggs by making the following crosses.

Catla♀× *Catla*♂ ⎫ CC

Rohu♀× *Rohu*♂ ⎭ RR

Catla♀ × *Rohu*♂ ⎫ Hybrid CR

Rohu♀× *Catla*♂ ⎭ Hybrid RC

Postlarvae of both hybrids and parental species were stocked in separate prepared earthen nursery ponds and after thinning reared up to the juvenile stage, average weight 25-30 g. At this stage, 300 seed of each cross-type (CC, RR, CR and RC) were transferred to Fisheries and Aquaculture Station, QAU, in plastic bags by adopting live hauling technique and after tempering, stocked separately in fiberglass tanks for acclimatization.

Experimental design

For comparative evaluation of disease resistance, innate immunity, temporal and spatial expression of immune related genes after injection of pathogenic bacteria, an

experiment was conducted in a replicate of two for each population. After about two-week acclimation 200 fish from each population were distributed evenly in their respective 8 tanks at a stocking density of 2g/L (25 fingerlings/tank). First two tanks of each cross-type were marked as a control without exposure to pathogen while the other six tanks were exposed to the pathogen. Among pathogen exposed tanks, 2 each were used for evaluation of cumulative mortality (%) during 20 days exposure, the other 2 for the evaluation of immunological indices and the last two for the temporal and spatial expression of immune related genes. Each fiberglass tank was well equipped with a proper aeration system for maintaining DO level and a water heater to maintain temperature. After one week of acclimatization in their respective tanks, all fingerlings except in two tanks of each cross-type were exposed to *Aromonas hydrophila*, a fresh culture of pathogenic bacteria. The suspension of bacteria was injected with 2ml syring (22 G needle) in the intraperitoneal cavity of the fish at the concentration of 2.24×10^7 CFU/g (Das et al., 2011). The bacteria strain was isolated from infected *L. rohita* by the Department of Microbiology, QAU, Islamabad. The control fish was injected 100µL sterile PBS. The experiment was conducted under control conditions (temperature, 27˚C, DO 6.0±0.5 mg/L, photoperiod, 14L and 10D). During the exposure period, all fish were fed once at 9:00 AM and to avoid water deterioration, fecal matter and un-consumed feed were removed daily through siphoning.

For evaluating disease resistance, the experiment was conducted for 20 days and in this duration, fish were keenly observed for symptoms of diseases and survival was noted. To verify that the mortality was due to the introduction of the pathogenic bacteria, the process of the autopsy was carried out and the *A. hydrophila* bacteria was isolated again from the kidney and skin of infected fish. The post challenge percent mortality and relative percent survival were calculated using formulas mentioned earlier (Amir et al., 2019).

For hematological indices, at random 18 fingerlings of each cross-type were collected from their respective control group (9 fingerlings/tank), and anaesthetized with buffered MS222 (0.1g/100mL) by immersing in the solution for 2 to 3-min. Following the cessation of opercula movement, the caudal vein was used for drawing blood from each fish by using 2 ml disposable syringes that were properly heparinized to avoid clotting and collected in K2 EDTA tube (BD Vacutainer®). The gauge size of the needle was 24. For taking enough volume, blood taken from one cross-type was placed in the same tube.

Evaluation of immunological indices was carried out 24 hrs post infection, blood of 18 fingerlings (9/tank), each from control and pathogen exposed groups of each cross-type (CR hybrid, RC hybrid, CC and RR) were collected by adopting the same procedure. Here also, the blood of two fish of the same group of the same cross-type was pooled and it was let to cool at room temperature. After about 60 min, the blood was centrifuged for 10 min at 2000 rpm and serum was separated and was collected in a tube and saved at a temperature of 4˚C till the analysis of immunological parameters. Fresh blood was used for the analysis of the respiratory burst and phagocytic activity.

For the gene expression, 6 fingerlings from the last two tanks of each population (3 fish/tank) were captured after 0, 6, 12, 24, 48 and 168 hrs post challenge, anaesthetized, dissected and their liver and muscle samples were collected and kept separately in autoclaved Eppendorfs containing RNA Later™ and stored at -80˚C till isolation of RNA.

Immunity parameters

The collected serum was used for the analysis of total serum proteins, immunoglobulin (IgM) and lysozyme activity through the respective standard methods used earlier (Ullah et al., 2018). The phagocytic and respiratory burst activity was measured from the fresh heparinized blood (Devi et al., 2019).

Hematological parameters

The blood parameters, counts of RBC (Red Blood Cell), Hb (Hemoglobin levels), MCV (Mean corpuscular volume), MCH (Mean cell hemoglobin), MCHC(mean corpuscular hemoglobin concentration), Hct percentage, Lymphocytes, Platelets (PL), Packed Cell Volume (PCV) were analyzed by using automated hematological analyzer (Model: Sysmex KX-21, Japan).

Lysozyme activity

For the analysis of blood serum lysozyme activity, Anderson and Siwicki (1995) method were used. To follow the given procedure, 0.9 ml of a 0.75 mg mL^{-1} *Micrococcus lysodeikticus* (Sigma-Aldrich, St Louis, MO, USA) suspension in phosphate buffered saline (pH 6.2) was mixed with 0.1 ml serum. The solution was thoroughly mixed and then the

absorbance change rate was observed at a wavelength of 450 nm and was noted after 1 min intervals for ten minutes by using a spectrophotometer (Model Micro-spectroAgilant 8453). The standard taken for the lysozyme activity was hen egg-white lysozyme (Sigma-Aldrich).

Total protein

For the analysis of total protein, Lowry et al. (1951) method was adopted for the assessment of total protein content in blood serum. For this purpose, 1 mg bovine serum albumin (BSA) in 1mL distilled water (1mg/1mL) was dissolved to prepare a stock solution. The various concentration of BSA (0.05 to 1 mg/ ml) was prepared by diluting the stock solution with distilled water. Subsequently, 200µL of each concentration was mixed with 2 ml of alkaline copper sulfate reagent and incubation was further carried for 10 min at ambient temperature. Then 0.2 ml of Folin's phenol reagent solution was added to each tube and incubation was done again for 30 min. Finally, optical density at a wavelength of 660 nm was calculated and plotted against the concentration of protein so that a standard calibration curve is obtained. A standard curve was used to know the protein concentration in unknown samples.

IgM level

To determine the serum level of immunoglobulin, the method of Anderson and Siwicki (1995) was followed. To execute the process, separation of immunoglobulin from the serum by the precipitation using polyethylene glycol (12%) was carried out, i.e., thorough mixing of 0.1 ml serum with 100 µl polyethylene glycol. Subsequently, the solution was incubated using an incubator shaker (Eppendorf Innova® 43/43R) under thorough shaking at room temperature for 120 min. After this, centrifugation at 7000 × g for 10 min was carried out. Supernatant from the centrifuged solution was then collected in a tube and its absorbance was noted at 660 nm wavelength. The protein content of the supernatant was subtracted from the total protein content in serum to calculate the IgM level.

Respiratory burst activity

The blood level of oxygen radicals production from the phagocytes was calculated through the application of a dye, nitroblue tetrazolium (NBT) as reported by Anderson and Siwicki (1995). To carry out the process, Blood (0.1 ml) was obtained and placed in an

Eppendorf tube (2 ml). Subsequently, the addition of 100 µl of 0.2% NBT was done. Incubation was carried out at ambient temperature for 30 min. Then, the removal of 0.05 ml of NBT-blood cell suspension was carried out and it was then added to a tube having 1 ml of N, N dimethylformamide. Centrifugation of the mixture at 3000× g for 5 min was done and the supernatant was obtained and placed in a glass cuvette and its OD was measured at a wavelength of 540 nm. Same items and procedures were followed but blood was only replaced with distilled water.

Phagocytic assays

Anderson and Siwicki (1995) method using *Staphylococcus aureus* for the detection of phagocytic activity(Sigma, St Louis, MO, USA) was carried out. To carry out the procedure, Heparinized blood (0.1 mL) was taken and placed on a microtiter plate well and 0.1 mL of killed *Staphylococcus aureus* 1×10^7 cells were added to it. The solution was suspended in phosphate buffered saline (0.1 ml) (pH 7.2). To mix bacteria with leucocytes thoroughly, the solution was properly mixed using a pipette. Incubation of the blood bacteria solution was carried out for 30 min at ambient temperature. After that, a smear was prepared by placing 5µL of the prepared solution on a clean glass slide. Subsequently, the glass slide was let to air dry and fixed using 95% ethanol for 5 min and air-dried once again. Giemsa stain (7%) dye was used to stain the smear for 10 min. A total of two smears were prepared from each fish. Observation under the light microscope was carried out and a total of 100 phagocytic cells including monocytes and neutrophils from each smear was observed. The number of phagocytic cells and phagocytosed bacteria were also observed and noted. 100 phagocytes of each slide were counted using a microscope for the determination of Phagocytic activation (PA) and phagocytic index (PI). The mean of each slide was calculated as below;

$$\mathrm{PA} = \frac{\text{No. of phagocytic cells having engulfed bacteria}}{\text{no. of phagocytes}} \times 100$$

(PA, % of cells having engulfed bacteria)

$$\mathrm{PI} = \frac{\text{No. of engulfed bacteria}}{\text{phagocytic cells}}$$

(PI, No. of engulfed cell/bacteria).

Isolation of RNA and cDNA synthesis

Muscles and liver tissues were used for the isolation of total RNA using a reagentTrizol using the given protocol and then subsequently stored at -20°C. NanoDrop (ND 1000) spectrophotometer (Thermo Fischer, USA) was used for the quantification of the purity, quality and concentration of the isolated RNA. Subsequently, total RNA from each sample was retro transcript to complementary DNA (cDNA). The total reaction volume was 20µL, contained 4µL of RNA, 2.5 4µL of Random primer, 14µL of MMLV-RT, 0.5 4µL of R Nasin, 3 4µL of H_2O, 4µL of Buffer and 1 µL of dNTPs. The reaction mixture was kept in the water bath at a temperature of 37°C for one hr and subsequently for 5 min at 55°C. Concentration and quality of cDNA were also assessed by using NanoDrop and subsequently preserved till use at -20°C.

Designing and validation of Primers

Self-designing of the primers to be utilized during the study was carried out through Oligo Primer Analysis Software version 1.1.2. Designing was done based on the sequences of mRNA of*L.rohita*available on gene bank. Table. 1 shows the sequence and annealing temperature. Expression of the required genes was done by quantitative real-time RT-PCR (qPCR). Validations of designed oligonucleotides were addressed by checking the efficiency of the PCR reaction through the generation of standard curves by the serial dilution of control samples of cDNA. The efficiencies were close to 100 %. Housekeeping gene 18-s was used both for the amount normalization and also as a positive control of mRNA in each sample.

RT-PCR (qPCR)

All amplification reaction consists of starting denaturation for 10 min at 95°C. The denaturation is followed by multiple cycles (40) for 15 sec at a temperature of 95°C followed by 62°C for 15 s. For qPCR reaction, a total 40 µL mixture consisted of a cDNA(3.2 µL), primer each of 1.6 µL, SYBER green master mix (20 µL) and 13.6 µL of H_2O was used. All qPCR assays were performed at a minimum of two runs in duplicate. In the negative control, water was added instead of cDNA. The mean threshold (Ct) value of each sample was used for analysis.

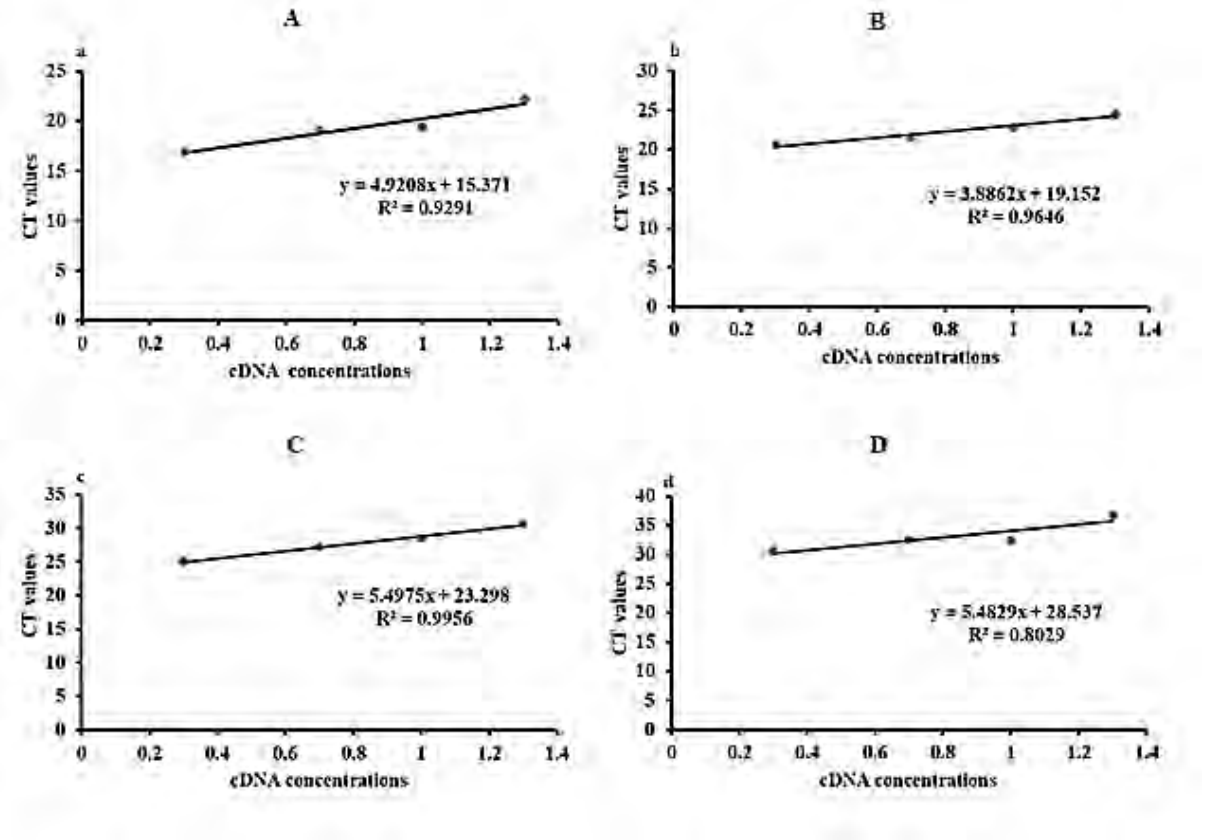


Fig.17. Linearity of housekeeping gene (A) 18s, (B) Lysozyme C, (C) Lysozyme G, and (D) TNF-α

Statistical analysis

The expression of each gene level was calculated using a relative quantification method (Pfaffl, 2001). Results are expressed as Mean ± SE. By using Statistic version 8.1, Statistical analysis tests (One way ANOVA followed by Duncan's multiple range) was applied for comparative assessment of pre and post-challenge immunological indices and relative temporal and spatial expression of immune related genes: Lysozyme C, Lysozyme G and TNF-α in the muscle and liver of all cross types (RR, CC, CR and RC). The level of significance was kept at 5%. Moreover, GraphPad Prism 5 software was used for plotting mean values along with their SE.

Results

Hematological parameters

Blood parameters values of the fingerlings of each cross-type recorded during this study are presented in (Table 21).One way ANOVA indicated significant effect of cross-type on the hematological indices, i.e., RBC (n=9, ANOVA, $F_{3,32}$=,16.6 $p<0.001$), WBC (n=9, ANOVA, $F_{3,32}$= 744, $p<0.001$), Hb (n=9, ANOVA, $F_{3,32}$=16.1, $p<0.001$), Hct (n=9, ANOVA, $F_{3,32}$=10.8, $p<0.001$), MCV (n=9, ANOVA, $F_{3,32}$= 29.7, $p<0.001$), MCH (n=9, ANOVA, $F_{3,32}$= 79, $p<0.001$) and MCHC (n=9, ANOVA, $F_{3,32}$= 146, $p<0.0001$) of fingerlings. Stastical analysis test (Post hoc LSD)showed that F1 fingerlings of RC followed by CR had the highest value of RBCs, WBC MCH, MCHC, HB and Hct% as compared to fingerlings of other cross-type while platelets and MCV levels showed low values in RC fingerlings as compared to other cross type.

Survival rates after *A. hydrophila* infection

During the 20 days after pathogen challenge, no mortality was observed in the fingerlings of control groups of all cross-types (RR, CC, RC, and CR), while *A. hydrophila* injected groups of all cross-type showed significant mortality. The survival rate analysis indicated a considerable difference ($p<0.001$) of survival (%) in the fingerlings of all cross-type. The pairwise comparison indicated the highest survival % of the fingerlings of RC (66.67 %) followed by CR cross-type (60 %) while CC cross-type fingerlings showed the highest survival rate (53.33 %) (Fig 18). Overall F1fingerlings of both RC and CR cross-type showed higher survival (resistance to the pathogen) as compared to fingerlings of purebred CC and RC cross-type

Immunological indices

Two way ANOVA test showed that there is a considerable difference in the immunological indices in the fingerlings of various cross-type (n=9, plasma protein, $F_{3,72}$=90.765, $p<0.001$; immunoglobins, $F_{3,72}$= 33.906, $p<0.001$; serum lysozyme activity, $F_{3,72}$= 53.948, phagocytic activity, $F_{3,72}$= 24.32, $p<0.001$; phagocytic index, $F_{3,72}$= 22.95, $p<0.001$, and respiratory burst activity, $F_{3,72}$= 23.35, $p<0.001$) before and after challenge to *Aeromonas hydrophilia* (n=9, plasma protein , $F_{1,72}$= 316.225 $p<0.001$), immunoglobins,

$F_{1,72}$= 78.632, p<0.001), serum lysozyme activity, $F_{1,72}$= 379.308, p<0.001; phagocytic activity, $F1_{,72}$= 13.23, p<0.001; phagocytic index , $F_{1,72}$= 61.97, p<0.001 and activity of the respiratory burst, $F_{1,72}$= 52.86, p<0.001) (Table 22). A statistical test (Post hoc LSD) showed the highest values of all studied immunity parameters: total protein (serum), IgM, lysozyme activity, respiratory burst activity, phagocytic activity and phagocytic index among the fingerlings of RC followed by CR while CC showed the lowest values. Moreover, fingerlings of all crosses had significantly higher values of all innate immunity parameters in the pathogen injected as compared to a control group. Overall, both hybrids showed higher immunity and showed heterosis (LPH, MPH and HPH) in all studied innate immunity parameters before and after challenged to *A. hydrophila* (Fig 19 and 20). Based on immunity, F1 fingerlings of all cross-type followed the following rank RC > CR >RR>CC before and after challenge to the pathogen, while based on heterosis, RC hybrid follow the CR hybrid.

Immune related genes expression

mRNA level of Lysozyme C

The lysozyme C periodic expression of the gene in the muscle and liver of the fingerlings of all cross-type (RR, CC, RC, and CR) after pathogen injection is shown in (Fig 22). Three ways ANOVA indicated that after challenging to *A. hydrophila*, the relative expression of Lysozyme C was significantly different over time ($F_{5,288}$=39.2, P< 0.001) in different tissues ($F_{1,\ 288}$=677.657, P< 0.001) among fingerlings of different cross-type (n=9, $F_{3,288}$=34, P< 0.001). The considerable interaction between time and cross-type ($F_{15,288}$= 2.58, P< 0.001), cross-type and tissue ($F_{3,288}$ = 6.08, P< 0.001), tissues and time (($F_{5,288}$ = 18.26, P< 0.001) and cross-type, tissue and time ($F_{15,288}$ =1.60, P= 0.074) indicated the manner, the temporal and spatial response of fingerlings of all cross-type varied. ANOVA followed by pairwise comparison of spatial mRNA level indicated increased lysozyme C expression in the liver compared to the muscle of all cross-type fingerlings, both before and after bacterial challenge. The comparative temporal expression in the liver indicated significantly higher expression at 6 -12 hr post-challenge followed by gradual down-regulation. However, muscles lysozyme C, mRNA level increased at 6hr after bacterial inoculation and remained higher for a longer period, i.e., up to 48 hr. The pairwise statistical analysis showed a significantly higher lysozyme C mRNA level in RC hybrid followed by

RR and CR while fingerlings of CC cross-type showed comparatively lower expression. This trend was found at all sampling periods. Both hybrids showed LPH in the basal (RC = >69%, CR=>26%) and post-challenge peak expression of lysozyme C in the liver (RC = >52%, CR=>13%), while only RC hybrid showed MPH (basal >44%, peak >83%, and HPH (basal = 25%, peak >30%). In muscle, again heterosis was observed in the expression of Lys C before and after challenge test, however, F1 RC hybrid showed relatively higher LHP, MPH and HPH as compared to F1 CR hybrid (Fig 25)

mRNA level of Lysozyme G

The spatial and temporal Lysozyme G gene expression in the fingerlings of all cross-type (RR, CC, RC, and CR) after injection of A. *hydrophila* is shown in (Fig23). Like Lys C, the Lys G also showed significantly different temporal ($F_{5,288}$=238.6, P< 0.001) and spatial expression ($F_{1, 288}$=1.365, P< 0.001) among fingerlings of different cross-type ($F_{3, 288}$=506.97, P< 0.001). The considerable interaction between time and cross-type($F_{15, 288}$=23.74, P< 0.001), cross-type and tissue ($F_{3, 288}$=42.66, P< 0.001), tissues and time ($F_{5, 288}$=70.18, P< 0.001) and cross-type× tissue × time ($F_{15, 288}$= 11.56, P< 0.001) indicated the manner, the temporal and spatial expression of Lysozyme G varied among the fingerlings of all cross-type. ANOVA followed by pairwise comparison of spatial mRNA level indicated higher expression of lysozyme G in the muscle as compared to the liver of all cross-type fingerlings, both before and after bacterial challenge. The comparative temporal expression in liver and muscle indicated significantly higher expression at 6 -12 hr post-challenge followed by gradual down-regulation. The pairwise comparison among cross-types indicated a significantly higher lysozyme G mRNA level in RC hybrid followed by CR and RR while fingerlings of CC cross-type showed comparatively lower expression. This trend was found at all sampling periods. Moreover, in the liver of RC hybrid, Lysozyme G attained peak expression earlier, i.e., at 6 hr as compared to 12 hrs, observed in the fingerlings of other cross-type, while in muscle all cross-type showed peak expression at 12 hrs post-challenge. Both hybrids showed LPH, MPH and HPH heterosis in the expression of Lys G in the liver and muscle. However, RC hybrids showed relatively higher levels of heterosis as compared to CR hybrid (Fig 25)

mRNA level of TNF-α

Temporal and spatial expression of TNF-α in the fingerlings of all cross-type after the infestation of a pathogen is shown in (Fig24). Three ways ANOVA indicated that the relative post-challenge expression of TNF-α varied significantly over time ($F_{5,288}$=622.31, P< 0.001) in different tissues ($F_{1, 288}$=92.731, P< 0.001) among fingerlings of different cross-type (n=9, $F_{3, 288}$=200.71, P< 0.001). The considerable interaction between time and cross-type ($F_{15, 288}$= 30.51, P< 0.001), cross-type and tissue (($F_{3, 288}$= 24.77, P< 0.001), tissues and time(($F_{5, 288}$= 87.02, P< 0.001) and cross-type× tissue × time ($F_{15, 288}$= 8.67, P< 0.001) indicated the manner, the temporal and spatial expression of TNF-α varied among the fingerlings of all cross-type. ANOVA followed by pairwise spatial expression of TNF-α indicated, increased expression in the muscle tissue compared to the liver of all cross-type fingerlings, both before and after bacterial challenge. The comparative temporal expression of TNF-α in the liver and muscle indicated a significantly higher expression at 6 -12 hr post-challenge followed by gradual down-regulation. The pairwise statistical analysis among cross-type showed a considerably higher TNF-α expression in the fingerlings of RC hybrid followed by CR and RR cross-type while fingerlings of CC cross-type showed comparatively lower expression. This trend was found at all sampling periods. Both hybrids showed LPH, MPH and HPH heterosis in the expression of TNF-α in the liver and muscle before and after challenged to *A.hydrophila*. However, RC hybrids showed relatively higher levels of heterosis as compared to CR hybrid (Fig 25)

Table 21. Hematological parameters of the fingerlings of all cross-types

	Cross-types			
	Purebred species		Hybrids	
Hematological indices	**RR**	**CC**	**CR**	**RC**
WBCs(10^3 µ/L)	222.6±1.60^{c}	197±0.92^{d}	250±1.53^{b}	281.6±1.13^{a}
PLT(10^3 µ/L)	20±0.91^{c}	38±1.07^{b}	46±1.68^{a}	22±1.75^{c}
RBCs(10^6 µ/L)	1.88±0.07^{b}	1.56±0.14^{c}	2.26±0.10^{a}	2.51±0.07^{a}
MCH (pg)	41.3±0.70^{b}	39.9±0.90^{c}	56.2±1.64^{a}	57.4±1.75^{a}
MCHC (g d/L)	26.56±0.46^{c}	25.29±0.24^{c}	32.6±0.41^{b}	35.6±0.45^{a}
MCV (fL) 10/L^{15}	159.3±1.64^{b}	174.92±1.60^{a}	157.81±1.29^{c}	157.14±1.68^{c}
HB (g d/L)	8.5±0.44^{c}	8.1±0.44^{c}	10.9±0.71^{b}	14.33±0.94^{a}
Hct (%)	33.50±0.99^{bc}	30.80±0.78^{c}	35.60±0.77^{b}	39.30±1.65^{a}

Hematological indices values presented as Mean (± SE). Sample size (n) = 9 for each cross-type. All means having different lowercase superscripts within a row are significantly different (P≤ 0.05). RR= R♀ × R♂; CC= C♀ × C♂; CR = C♀ × R♂ and RC= R♀ × C ♂.

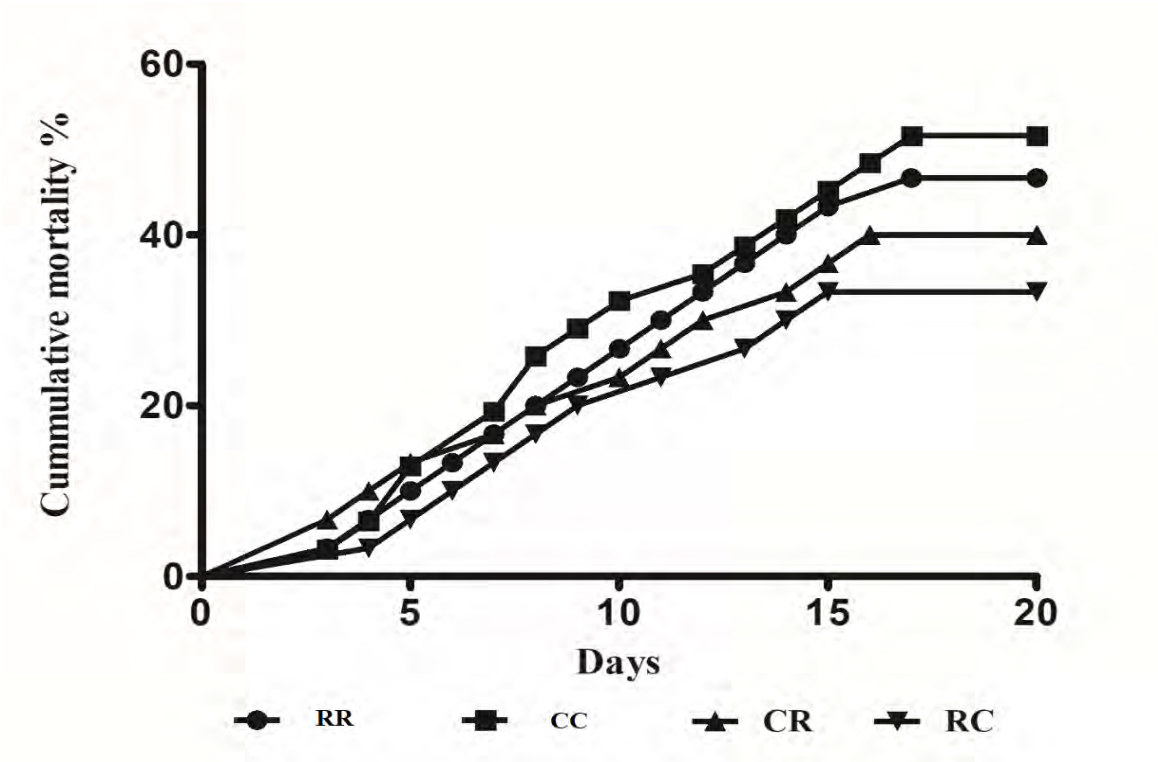


Fig. 18. Cumulative mortality (%) of fingerlings of all cross-types after the infestation of *A. hydrophila.* RR= R♀ × R♂, CC= C♀ × C♂, CR = C♀ × R♂ and RC= R♀ × C ♂.

Table 22. Immunological parameters of the fingerlings of all cross-types before and after challenge to pathogen

Immunological indices	Cross-types			
	Purebred species		Hybrids	
	RR	CC	CR	RC
Total Protein (mg /mL)				
Control	15.55 ± 0.42^{c}	12.47 ± 0.82^{d}	17.01 ± 0.39^{b}	23.75 ± 0.80^{a}
Challenged	$23.13\pm0.67^{C***}$	$21.08\pm0.58^{D***}$	$25.99\pm0.48^{B**}$	$29.03\pm0.51^{A**}$
Lysozyme (µg m/L)				
Control	2.91 ± 0.25^{b}	1.72 ± 0.29^{c}	3.50 ± 0.19^{ab}	4.05 ± 0.43^{a}
Challenged	$6.05\pm0.3^{C***}$	$4.98\pm0.32^{C**}$	$7.79\pm0.21^{B**}$	$9.93\pm0.30^{A***}$
IgM(mg m/L)				
Control	8.93 ± 0.44^{b}	5.93 ± 0.44^{c}	9.99 ± 0.88^{ab}	10.50 ± 0.68^{a}
Challenged	$12.04\pm0.51^{B***}$	$8.58\pm0.51^{C**}$	$14.98\pm0.78^{A**}$	$16.48\pm0.8^{A***}$
Phagocytic Activity %				
Control	51.03 ± 1.32^{ab}	39.61 ± 3.15^{b}	55.44 ± 2.48^{ab}	61.35 ± 3.29^{a}
Challenged	$52.57\pm1.74^{B*}$	$42.65\pm1.76^{C*}$	$55.66\pm2.66^{A*}$	$62.71\pm7.45^{A**}$
Phagocytic index				
Control	1.97 ± 0.20^{a}	1.03 ± 0.12^{b}	2.09 ± 0.03^{a}	2.38 ± 0.39^{a}
Challenged	$3.92\pm0.17^{B***}$	$2.78\pm0.19^{C***}$	$4.51\pm0.30^{AB***}$	$5.22\pm0.36^{A***}$
Respiratory Burst activity				
Control	0.43 ± 0.07^{d}	0.33 ± 0.05^{c}	0.63 ± 0.12^{b}	0.81 ± 0.05^{a}
Challenged	$0.75\pm0.09^{C*}$	$0.65\pm0.05^{C***}$	$0.95\pm0.5^{B***}$	$1.13\pm0.5^{A***}$

Innate immunity parameters of all cross-types before and after challenge to pathogen (A) Lysozyme (B) Serum protein (C) IgM (D) Respiratory burst activity (E) Phagocytic activity (F) Phagocytic index. Data bars show the values as Mean (± SE). Sample size (n) =9 for each cross-type. ANOVA followed by LSD shows a comparison in the immunity parameters values among fingerlings of all cross-types before challenge (lowercase superscripts) and after challenge (uppercase superscripts) to a pathogen. T-test compares the values within the cross-type before and after infestation. * = $P < 0.05$; ** = $P < 0.01$;*** = $P < 0.001$. RR= R♀ × R♂, CC= C♀ × C♂, CR = C♀ × R♂ and RC= R♀ × C ♂.

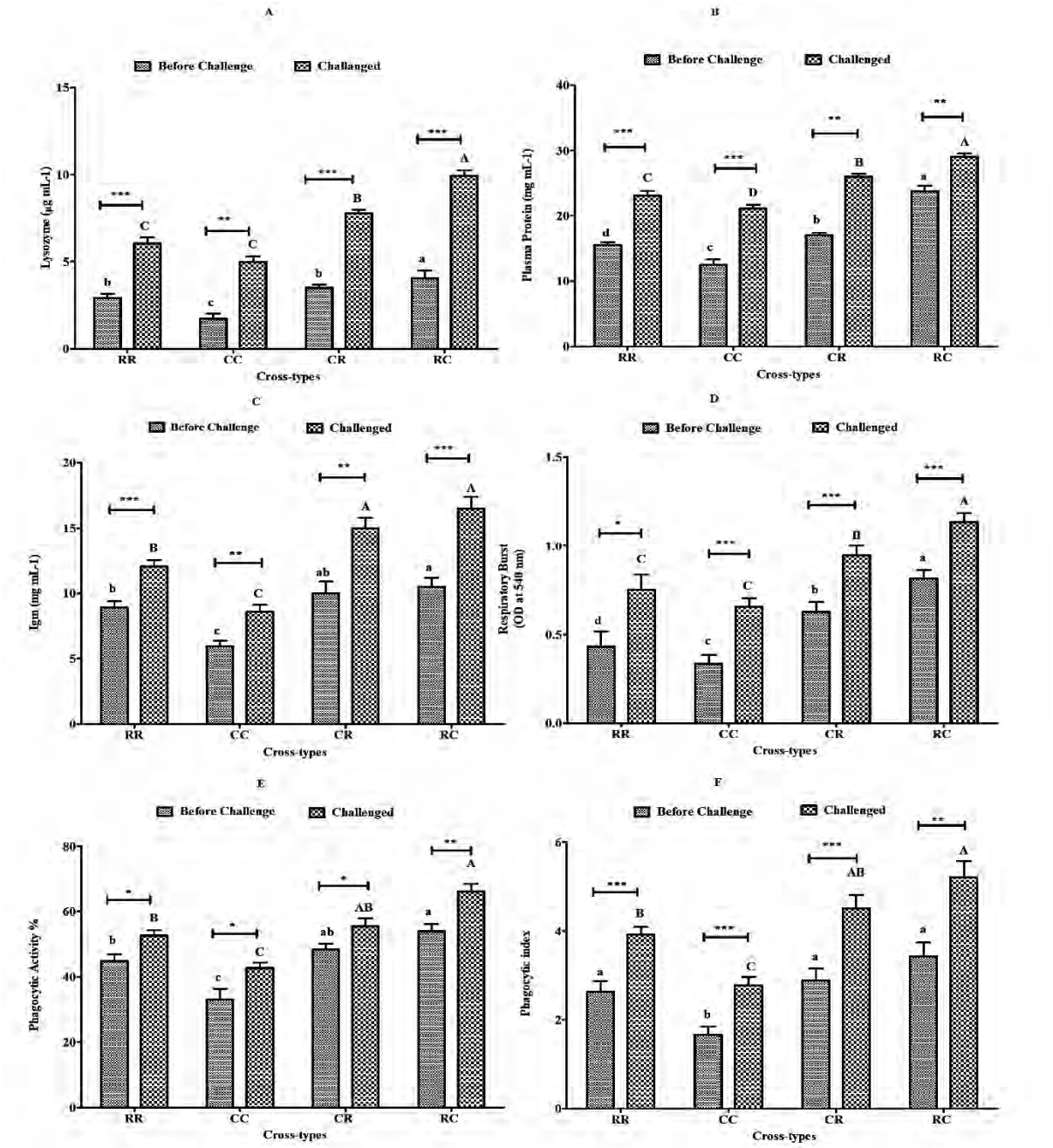


Fig. 19. Comparison of immunity parameters before and after challenge to pathogen (A) Lysozyme, (B) plasma protein, (C) IgM (D) respiratory burst activity, (E) phagocytic activity, (F) phagocytic index. The data in the bars show the values as Mean ± SE (n=9). ANOVA followed by LSD shows a comparison in the immunity parameters values between fingerlings of all cross-types before challenge (lowercase superscripts) and after challenge (uppercase superscripts) to a pathogen. T-test compares the values within the cross-type before and after infestation. (*** = P < 0.001;** = P < 0.01; * = P < 0.05; ns = non-significant. RR= *L. rohita*♀×*L. rohita*♂, CC= *C. catla*♀×*C. catla*♂, CR = *C. catla*♀×*L. rohita*♂, RC= *C. catla*♂ ×*L. rohita*♀.

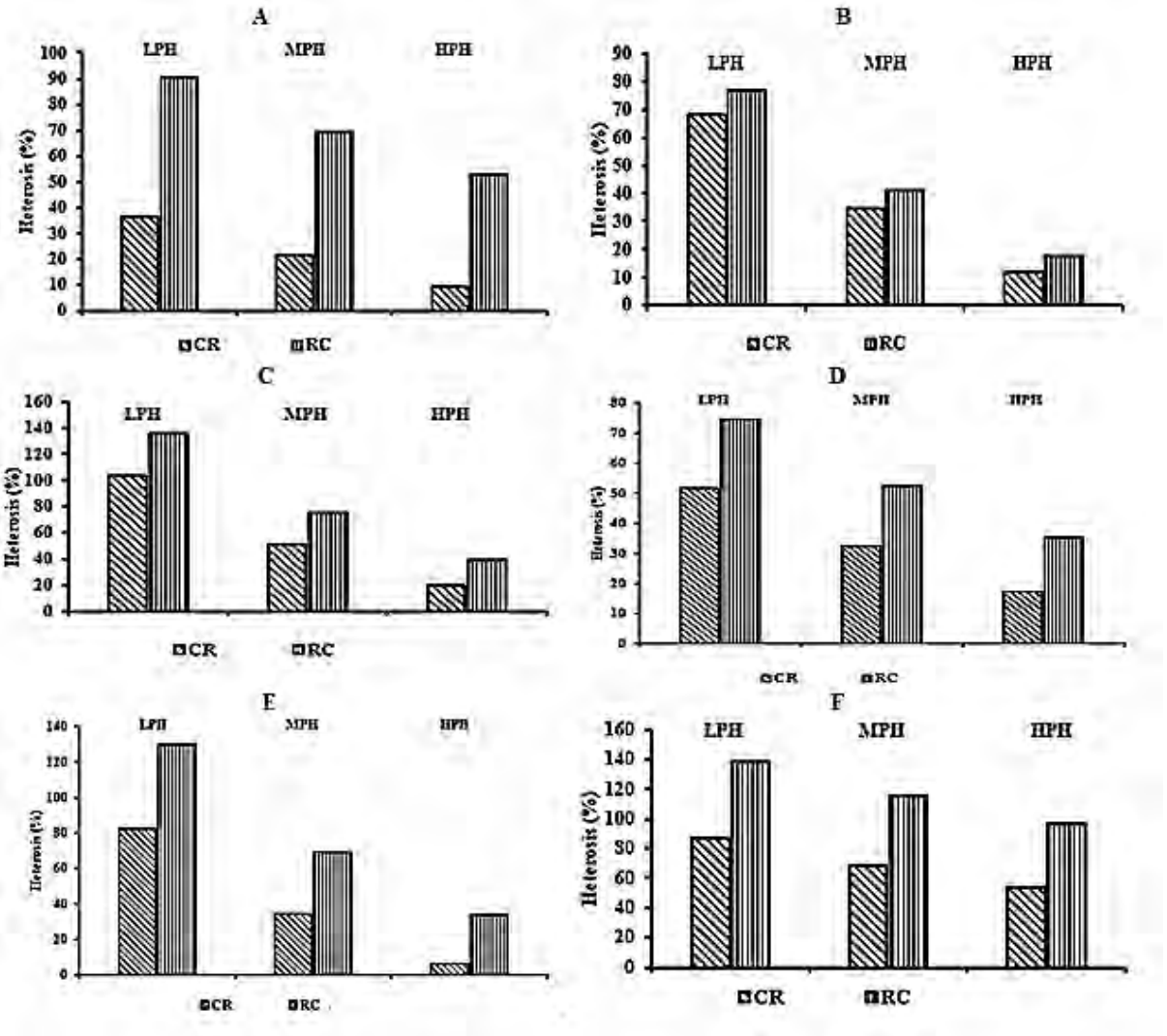


Fig. 20. Heterosis (%) in immunity parameters of the reciprocal hybrids of *C. catla* and *L. rohita* (A) serum protein, (B) IgM, (C) lysozyme, (D) phagocytic activity, (E) phagocytic index, (F) respiratory burst activity. CR = C♀ × R♂ and RC= R♀ × C. LPH= low parent heterosis, MPH= mid parent heterosis, HPH = high parent heterosis

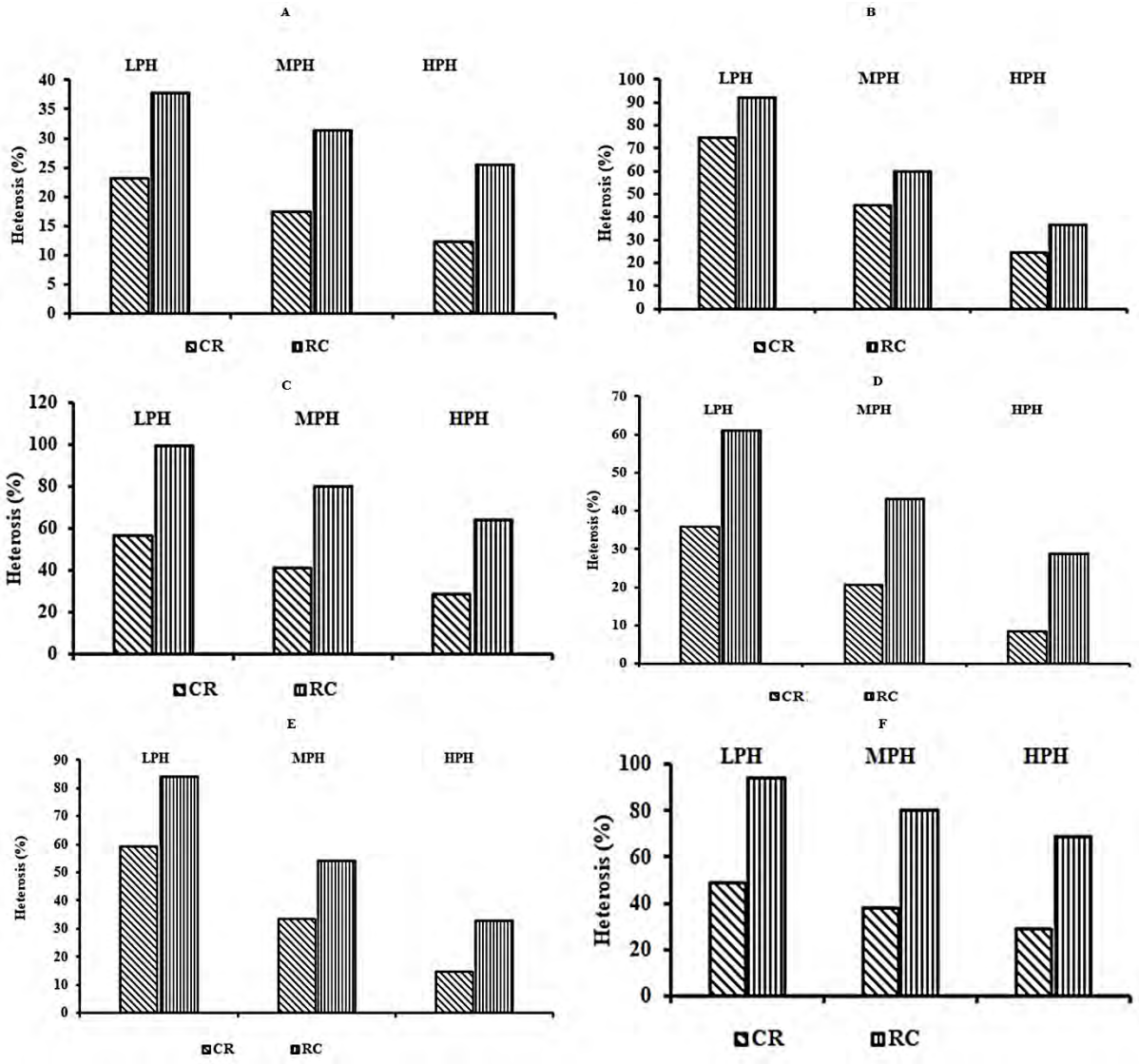


Fig. 21. Heterosis (%) in immunity parameters of the reciprocal hybrids of *C. catla* and *L. rohita* after infestation. (A) serum protein, (B) IgM, (C) lysozyme, (D) phagocytic activity, (E) phagocytic index, (F) respiratory burst activity. CR = C♀ × R♂ and RC= R♀ × C ♂. HPH = high parent heterosis. LPH= low parent heterosis. MPH= mid parent heterosis

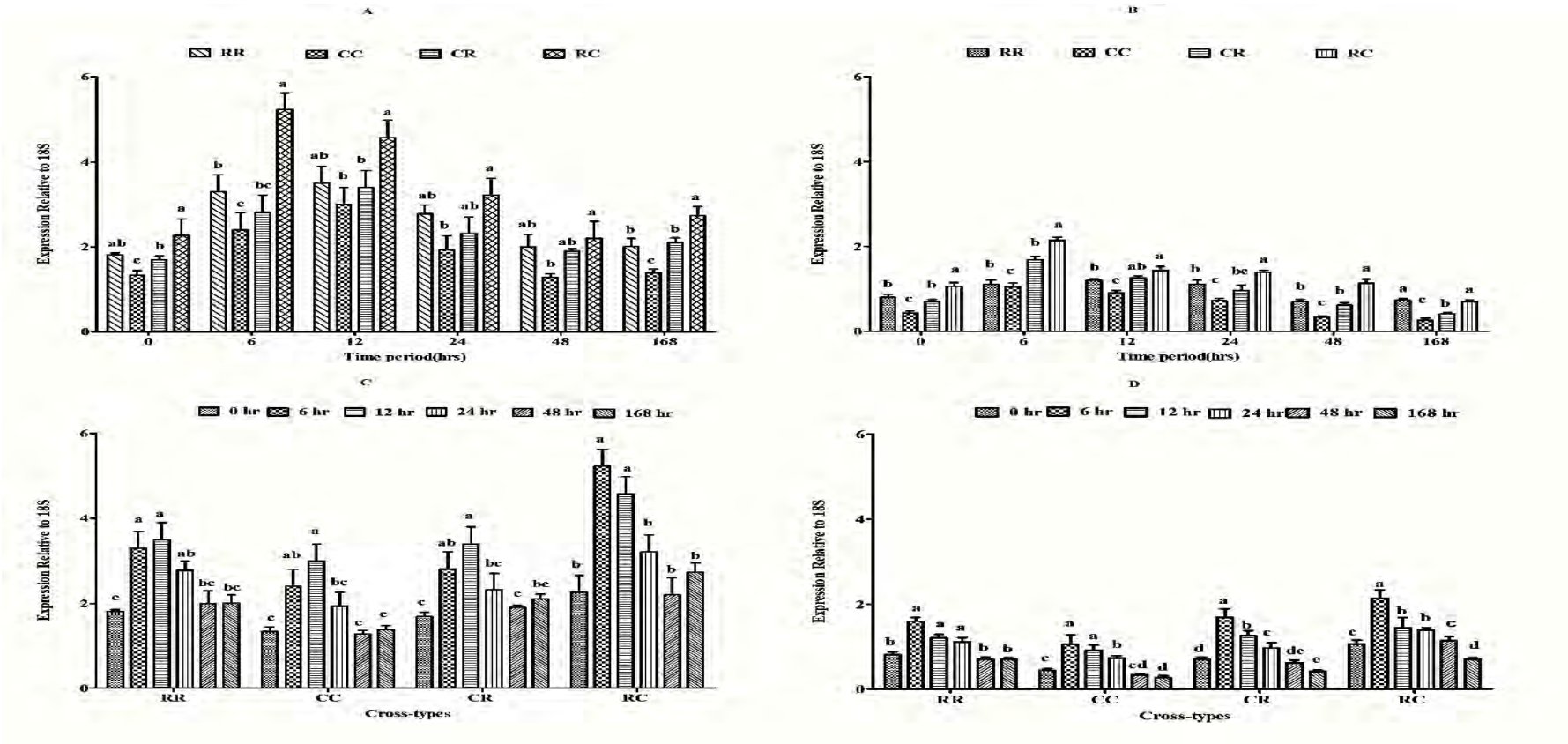


Fig. 22. Spatial and temporal variations in the mRNA levels of Lysozyme C gene among cross-types (A & B) and within cross-type (C & D) after the infestation of *A. hydrophila*. Liver (A & C), muscle (B & D). Data bars show the values as Mean (± SE). Sample size (n) = 6. All means having different lowercase superscripts on the bar are significantly different (P< 0.05). Different lowercase superscripts on bars are showing a significant difference (P < 0.05) within cross-type and between cross-types. RR= R♀ × R♂, CC= C♀ × C♂, CR = C♀ × R♂ and RC= R♀ × C ♂.

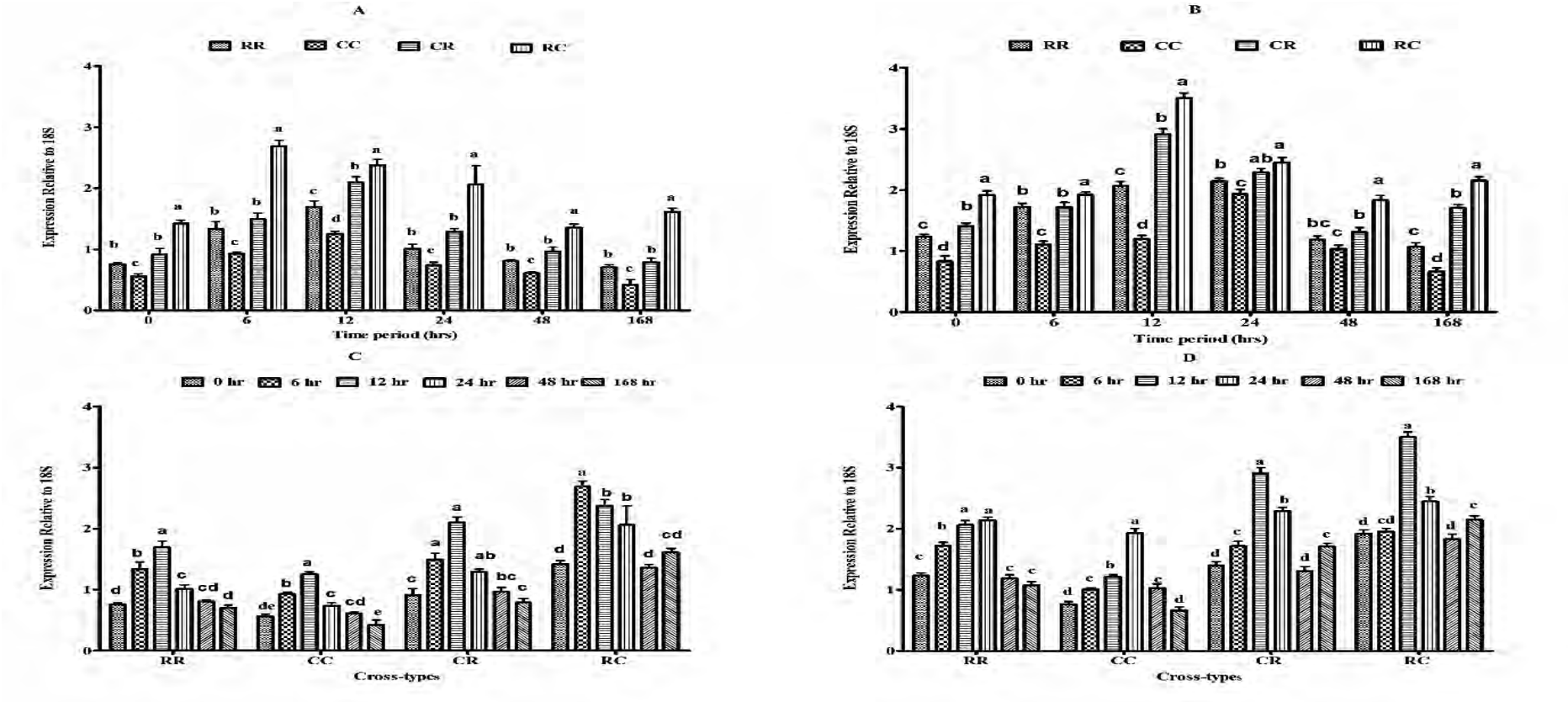


Fig. 23. Spatial and temporal variations in lysozyme G gene expression among cross-types (A & B) and within cross-type (C & D) after the infestation of *A. hydrophila*. Liver (A & C), muscle (B & D). Data bars show the values as Mean (± SE). Sample size (n) = 6. All means having different lowercase superscripts on the bar are significantly different (P< 0.05). Different lowercase superscripts on bars are showing a significant difference (P < 0.05) within cross-type and between cross-types. RR= R♀ × R♂, CC= C♀ × C♂, CR = C♀ × R♂ and RC= R♀ × C♂.

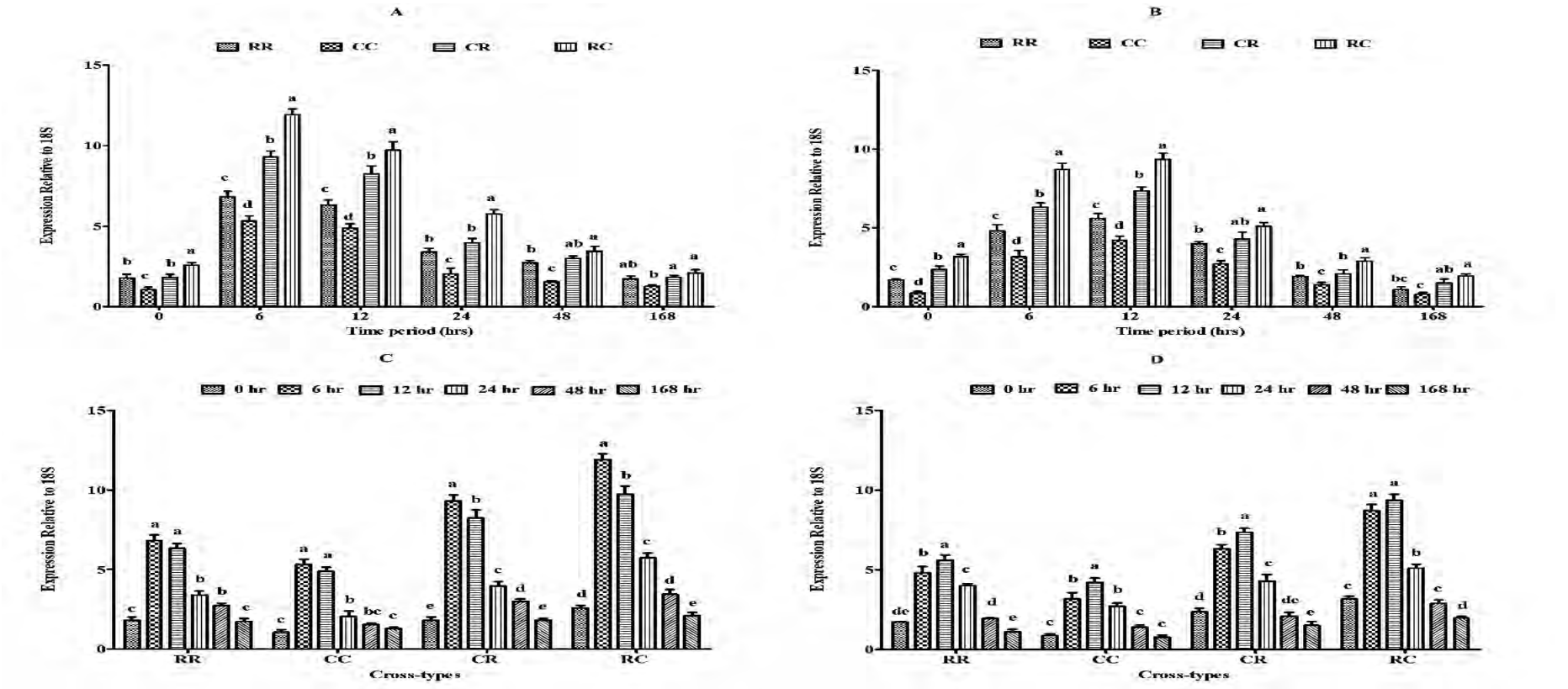


Fig. 24. Spatial and temporal variations in the expression of TNF-α gene, (A & B) among cross-types and (C & D) within cross-type after infestation of *A. hydrophila*. Liver (A & C), muscle (B & D). Data bars show the values as Mean (± SE). Sample size (n) = 6. All means having different lowercase superscripts on the bar are significantly different (P< 0.05). Different lowercase superscripts on bars are showing a significant difference (P < 0.05) within cross-type and between cross-types. RR= R♀ × R♂, CC= C♀ × C♂, CR = C♀ × R♂ and RC= R♀ × C♂.

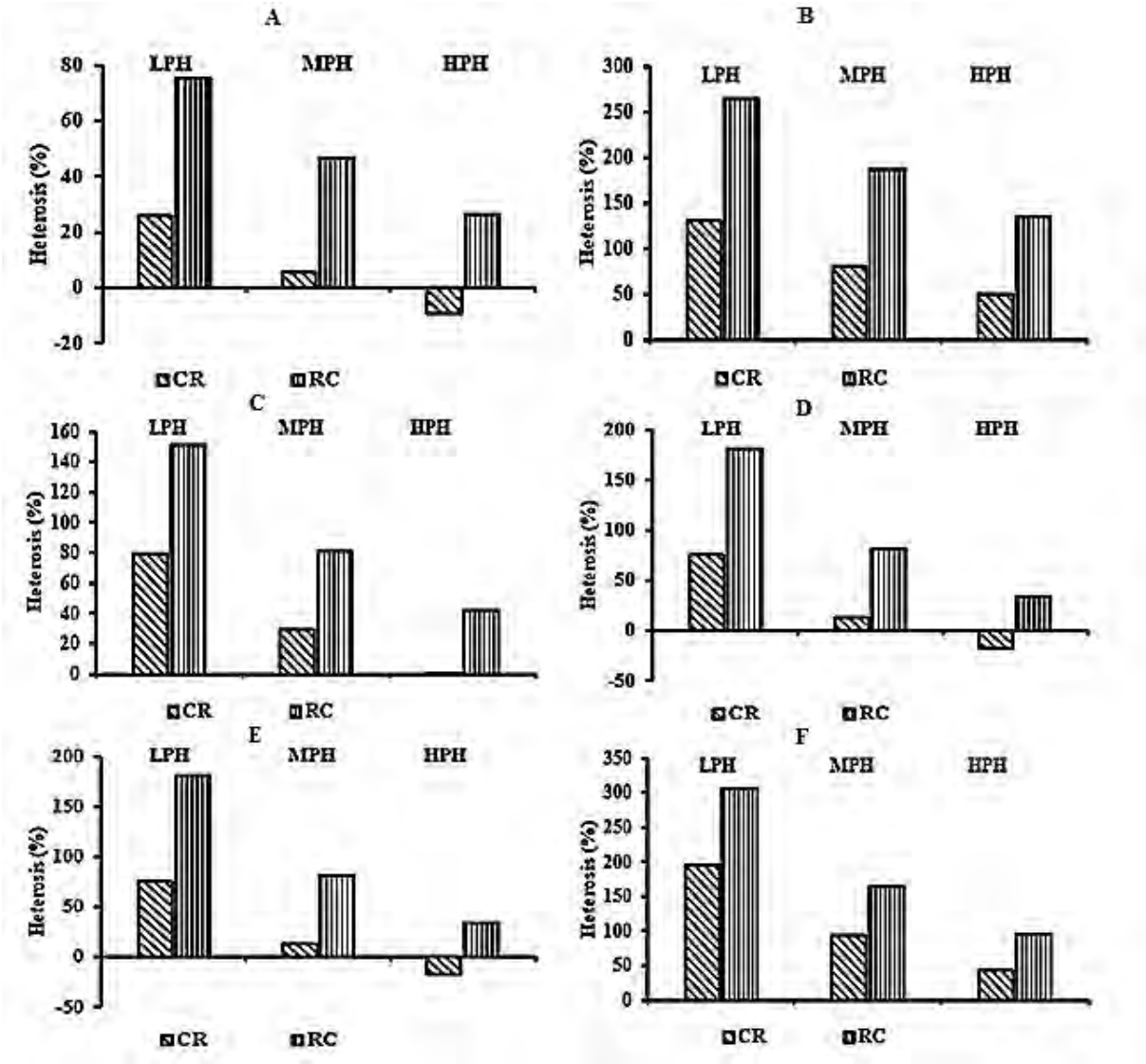


Fig. 25. Heterosis (%) in immunity genes parameters of the reciprocal hybrids of *C. catla* and *L. rohita* in liver. (A) Lysozyme C, (B) Lysozyme G, (C) TNF-α and muscle, (D) lysozyme C, (E) lysozyme G, (F) TNF-α. CR = C♀ × R♂ and RC= R♀ × C ♂. HPH = high parent heterosis. LPH= low parent heterosis. MPH= mid parent heterosis.

Chapter 4

Comparative Nutritive Values of *Labeo rohita, Catla catla* and their intergeneric hybrids

Materials and Method

Sampling

Healthy fish of each cross-type, with no visible sign of infection, body weight range 310-350g, were collected to evaluate the proximate composition, fatty acid, and amino acid profile of muscle. These fish were reared in earthen communal ponds in a semi-intensive culture condition on a 32% crude protein diet. After harvesting, fish were cleaned, degutted at a spot and kept in an icebox.

Proximate Analysis of muscle

To estimate the proximate composition of muscle, randomly 27 fish of all cross-types were collected from each pond (n= 9 per cross-type). To get enough sample, the carcass of the same cross-type fish was pooled (3 sample /cross-type) and analyzed by using NIR Analyzer (Foss Swiss)

Amino acid analysis

A high-performance liquid chromatography (HPLC) based amino acid analyzer (CBM-20A, Shimadzu, Japan), available at Medicinal botanical centre of Pakistan council for scientific research (PCSIR) Laboratory, Complex, Peshawar, was used for the amino acid profile.

Extraction of amino acid

For the analysis of amino acids, 10 fish of each cross-type from each tank (30 fish/cross-type) were collected. Fish were degutted, and the muscle of each fish was aseptically separated. For obtaining a sufficient sample, muscles of 10 fish from the same tank were pool (3samples/cross-type) and stored at -20 °C until further use.

For the extraction of amino acids, muscle tissue samples were thawed and 50 g muscle of each sample was dried to a constant weight in a vacuum oven. Dried muscle tissues were ground using a glass pestle motor and transferred to a dried glass tube. Subsequently, 5 ml HCl (0.1 %) was added and the test tube was shaken vigorously for 2-3 min and

centrifuged at 3500 rpm for 15 min. The supernatant was collected and filtered through a membrane filter (0.45µm) and used for amino acids profile.

Preparation of reaction solutions

To prepare the borate buffer, 40.7g of Na_2CO_3 and 13.57g of H_3BO_3 was dissolved in 600 ml of sterilized double-distilled water. The solution was mixed thoroughly to make a clear solution.

Solution A

The solution was prepared by mixing 0.2 ml of Sodium Hypochlorite solution (7-10% NaClO) with 500 ml of prepared buffer. The solution was filtered by passing through a membrane filter (pore size, 0.45µm).

Solution B

This solution was prepared by dissolving 0.4 g Ortho-Phthalaldehyde (OPA) in 7 ml ethanol and mixed with 450 ml of the prepared borate buffer solution. In this mixture 0.5 g, N. Acetyl Cysteine was added and shaken vigorously to make a homogeneous solution. To remove impurities resultant solution was filtered through a membrane filter (pore size, 0.45µm).

Preparation of mobile phase 'A'

Citrate tribasic (19.6 g) was dissolved in 600 ml of deionized water and then mixed with 70 ml of ethanol and 7.8 mL per-chloric acid. After mixing, the volume was raised to 1 L by adding double deionized water, and the pH of the mobile phase was adjusted (pH 3.2) by adding the perchloric acid.

Preparation of mobile phase 'B'

Citrate Tribasic (58.8 g) and boric acid (12.4 g) was dissolved in 800 ml of double-distilled water. After this 25 ml of NaOH (4 N) was added and volume was raised to 1L. The pH of the solution was adjusted (pH 10) by using NaOH and filtered.

Preparation of mobile phase 'C'

Sodium hydroxide (0.2 N) solution was prepared by dissolving 8 g of Sodium hydroxide in double-distilled water (1L) and filtered. Afterwards, 20 µl samples, was injected into HPLC based amino acid analyzer (Shimadzu, Japan). The column used for the amino acid profile was sodium-based with a fluorescent detector. Fluorescence was detected at excitation and emission wavelength of 350nm and 450nm respectively. Amino acids were separated by using mobile phase A and B. After separation, they were spelt out from the column where reaction solution (solution A and B) was combined with mobile phase (containing amino acids) and initiated the post-column derivatization. After derivatization, the complex was passed to the cuvette for the detection of different amino acids.

Fatty acid analysis

Fatty acids were analyzed from muscle tissue using Gas chromatography-mass spectrometry (GC-MS) at the medicinal botanical centre of Pakistan council for scientific research (PCSIR) Laboratory, complex Peshawar. For the analysis of fatty acids, 10 fish from each tank (30 fish/cross-type) were collected and the muscle of each fish was aseptically separated. For obtaining enough samples, muscles of 10 fish from the same pond were collected in the same tube (3 samples/treatment). Muscles were immediately frozen in liquid nitrogen and stored at -20 °C until further use.

Extraction of total lipid

The extraction of total lipid, preparation of fatty acids methyl esters (FAMEs), and composition of FAMEs were analyzed by adopting the procedure reported earlier (Afridi et al., 2018).

Extraction of oil from fish muscle

The desired amount of oil for fatty acid analysis through GC-MS was extracted from fish muscle tissues by using Soxhlet's apparatus. Briefly, 20g muscle tissues were dried in a Vacuum drying oven (Lab Tech Korea) for three days at 50˚C. Then the dried sample was used for the extraction of lipid. The dried sample was crushed and 5 g was transferred in a conical flask of Soxhlet apparatus. Subsequently, n-hexane (Fisher Scientific, UK) was added

and the flask was heated for about 7 hrs. Then extracted oil was condensed by using a rotary vacuum evaporator and shifted to glass vials. The Extra hexane was evaporated by adopting Poitevin (2016) method, i.e., to keep vials overnight at ambient temperature

Sample preparation for fame's analysis on GC- MS

Fatty acids methyl esters (FAMEs) were prepared and composition was analyzed by adopting the procedure reported earlier (Afridi et al., 2018). Briefly, FAMEs solution was prepared and after passing through membrane filter paper (Whatman pore size, 0.45um), 1µL solution was injected into Gas chromatograph-mass spectrometer (GC-MS-2010 Shimadzu Japan) having CP Sil 88 capillary column (length, 100 m (film thickness, 0.20 µm; i.d, 0.25 µm, Chrompack, Middleburg, Netherlands) and a flame ionization detector. The program for the lipid profile consisted of initial column temperature 80˚C, after I min increased at the rate of 2 °C/min until reached 215 °C, then maintained this temperature for 30 min. The Inlet and detector temperatures were set at 220°C and 230°C respectively, while a split mode of injection (100:1) was used. The H_2 carrier gas was flow at the rate of 1 mL/min. For the identification and quantification of fatty acids, either a pure FAME standard or quantitative mixture (Mix SUPELCO, CRM47885, Bellefonte, USA) containing 37 components was used. For the analysis of fatty acids, the chem station software and a data processor was used.

Results

Proximate analysis

Proximate muscle composition of fish of all cross-type is presented in (Table 23). One way ANOVA showed significant difference in moisture content (n=3, $F_{3,8}$=149, p<0.001), crude protein (n=3, $F_{3,8}$=126, p<0.001), fat content (n=3, ANOVA, $F_{3,8}$=34.7, p<0.001) and ash content (n=3, $F_{3,8}$=22.3, p<0.001) among the fish of all cross-type. The post hoc pairwise comparison indicated a significantly higher percentage of crude protein and fat in the muscle composition *L. rohita* followed by both hybrids, while *C. catla* showed a significantly higher concentration of moisture and Ash contents

Muscle amino acid (AA) composition:

The muscle amino acid profile of all cross-type is shown in (Table 24). One way ANOVA showed a significant effect of cross-type on the fish muscle amino acids composition, i.e., essential amino acids (n=3, $F_{3,8}$=838.02, p<0.001) and nonessential amino acids (n=3, $F_{3,8}$=526.80, p<0.001) concentrations. The post hoc pairwise comparison indicated the highest amounts of non-essential amino acids (>92%) in the muscle of *C. catla* followed CR hybrid (>89%), while RC hybrid showed the lowest value. However, muscle of RC hybrid contained a significantly the highest concentration of essential amino acids (EAA) (≈ 38%) especially alanine (≈21%) followed by *L. rohita* (>15%), while *C. catla* showed the lowest amount (7%).

Muscle Fatty acid composition

Muscle fatty acid profile of all cross-type is shown in (Table 25). One way ANOVA showed significant differences in muscle fatty acid composition, i.e., ∑saturated fatty acid(n=3,$F_{3,8}$= 88.41, p<0.001), ∑Monounsaturated fatty acids (n=3, $F_{3,8}$=72.17, p<0.001), ∑Polyunsaturated fatty acids (n=3, $F_{3,8}$=691.65, p<0.001) and ω3 to ω6 ratio (n=3, $F_{3,8}$=31.4, p<0.001) among cross-type. The post hoc LSD test indicated the highest concentration of MSFA (27.45%) and PUFA (>33%) in *L. rohita* (RR cross-type) followed by both hybrids while *C. catla* muscle composition showed the lowest values of MSFA and PUFA. The comparison among hybrids muscle fatty acid composition showed a statistically similar concentration of MUFA (p>0.05) and a significantly higher value of PUFA (p<0.05) in the

CR hybrid. Moreover, both hybrids (RC and CR) showed a relatively lower ratio of ω3 to ω6 PUFA as compared to purebred species (*L. rohita* and *C. catla*).

Table 23. Proximate muscle composition of *L. rohita C. catla* and their intergeneric hybrid produced from reciprocal crosses

	Cross-types			
	Purebred species		Hybrids	
Composition (%)	**RR**	**CC**	**CR**	**RC**
Moisture	72.31±0.29^{c}	79.05±0.23^{a}	76 .50±0.20^{ab}	75.15±0.58^{b}
Crude protein	19.13±0.20^{a}	14.50±0.21^{d}	15.37±0.15^{c}	16.50±0.15^{b}
Fat	4.21±0.06^{a}	2.67±0.20^{d}	3.27±0.06^{c}	3.71±0.12^{b}
Ash	1.30±0.1^{c}	2.0±0.01^{a}	1.8±0.01^{ab}	1.6±0.01^{b}
NFE	2.90±0.24^{a}	1.76±0.06^{b}	2.14±0.10^{b}	2.78±0.05^{a}

Proximate composition data are shown as Mean (± SE0. Sample size (n) =9 for each cross-type. All mean values sharing different lowercase superscript within a row are significantly different ($P < 0.05$). RR= R♀ × R♂; CC= C♀ × C♂; CR = C♀ × R♂ and RC= R♀ × C ♂. NFE= nitrogen-free extract

Table 24. Muscle amino acid composition of all cross-types

	Cross-types			
	Purebred species		Hybrids	
	RR	**CC**	**CR**	**RC**
Thr	4.77±0.07^{b}	4.93±0.59^{b}	5.83±0.01^{ab}	6.64±0.52^{a}
Gly	9.07±0.87^{b}	9.65±0.33^{b}	13.08±0.64^{a}	9.39±0.59^{b}
Val	3.72±0.17^{d}	9.30±0.91^{b}	6.67±0.17^{c}	11.03±0.18^{a}
Met	2.54±0.02^{d}	9.47±0.60^{a}	3.94±0.02^{c}	8.01±0.02^{b}
Lie	2.43±0.03^{c}	8.62±0.55^{a}	3.75±0.03^{b}	3.06±0.04^{bc}
Leu	2.57±0.02^{c}	2.70±0.02^{c}	5.76±0.12^{a}	3.06±0.04^{b}
Tyr	0.37±0.02^{c}	2.29±0.02^{b}	2.40±0.61^{b}	4.82±0.61^{a}
Phe	6.65±0.30^{b}	7.60±0.50^{b}	3.73±0.04^{c}	12.31±0.61^{a}
His	7.33±0.60^{a}	3.58±0.02^{c}	1.10±0.03^{d}	5.83±0.03^{b}
Lys	27.08±1.42^{a}	23.34±1.04^{b}	10.26±0.53^{c}	12.51±0.62^{c}
NH3	7.97±0.24^{a}	7.75±0.59^{a}	2.50±0.04^{b}	7.82±0.60^{a}
Arg	7.49±0.55^{a}	4.14±0.02^{c}	1.56±0.03^{d}	5.78±0.04^{b}
∑AA	81.35±0.48^{c}	92.65±0.46^{a}	60.55±0.50^{d}	89.20±0.55^{b}
Non-essential amino acids (NEAA)				
Ala	4.54±0.33^{b}	3.87±0.02^{b}	21.36±1.07 ^{a}	4.07±0.07^{b}
Asp	1.05±0.02^{b}	1.22±0.10^{b}	3.91±0.51 ^{a}	1.64±0.31^{b}
Ser	9.31±0.57^{b}	1.93±0.33^{d}	12.27±0.81^{a}	4.67±0.42^{c}
∑NEAA	15.38±0.56^{b}	7.0±0.12^{d}	38.10±1.16^{a}	10.73±0.34^{c}

Muscle protein amino acids are shown as Mean (± SE). Sample size (n) =9 for each cross-type.. All mean values sharing different lowercase superscripts within a row are significantly different ($P < 0.05$). RR= R♀ × R♂; CC= C♀ × C♂; CR = C♀ × R♂ and RC= R♀ × C ♂. Thr: threonine, Met: methionine; Gly: glycine, Val: valine Lie: isoleucine, Leu: leucine, Tyr: tyrosine, Glu: glutamic acid Phe: phenylalanine, Lys: lysine, His: histidine, Arg: Arginine, Ala: alanine, Asp: aspartic acid, Ser: serine

Table 25. Muscle fatty acid composition of all cross-types

Fatty Acids	No. of carbon atom	Cross-types: Purebred species RR	Purebred species CC	Hybrids CR	Hybrids RC
Myristic acid	14:00	2.21±0.15^{b}	3.35±0.29^{a}	1.69±0.12^{c}	1.84±0.03^{c}
Pentadecanoic acid	15:00	1.77±0.14^{c}	4.54±0.20^{a}	2.56±0.23^{b}	1.33±0.02^{c}
Palmitic acid	16:00	22.37±0.40^{c}	30.65±0.59^{a}	27.66±0.61^{b}	24.57±0.42^{c}
Margaric acid	17:00	2.56±0.20^{a}	1.83±0.09^{b}	1.40±0.12^{b}	2.12±0.01^{b}
Stearic acid	18:00	6.40±0.48^{c}	10.16±0.31^{a}	7.19±0.61^{c}	8.47±0.34^{bc}
Docosanoic acids	22:00	0.23±0.01^{c}	0.36±0.01^{a}	0.28±0.01^{b}	0.20±0.01^{c}
∑Saturated		37.34±3.66^{c}	51.22±.91^{a}	40.68±0.23^{b}	38.52±0.73^{bc}
Heptadecenioc acid	17:01	7.56±0.20^{b}	6.27±0.01^{c}	6.99±0.20^{c}	8.43±0.22^{a}
Oleic acid	18:1(n-9)	19.24±0.37^{a}	12.24±0.29^{d}	16.20±0.27^{b}	13.94±0.31^{c}
Eicosenoic acid	20:01	0.61±0.01^{c}	0.66±0.01^{b}	0.51±0.01^{d}	0.79±0.01^{a}
∑Monounsaturated		27.41±0.48^{a}	19.17±0.28^{c}	23.70±0.45^{b}	23.16±0.51^{b}
Linoleic acid (ω6)	18:2(n-6)	7.23±0.01^{c}	6.26±0.102^{d}	8.82±0.32^{b}	9.58±0.13^{a}
g-linolenic acid (ω6)	18:3(n-6)	0.27±0.02^{a}	0.16±0.01b^{c}	0.14±0.01^{c}	0.19±0.01^{b}
Linolenic acid (ω3)	18:3(n-3)	3.41±0.14^{a}	1.54±0.20^{c}	1.15±0.06^{c}	2.41±0.18^{b}
Eicosatrienoic acid (ω3)	20:3(n-3)	0.92±0.01^{a}	0.56±0.01^{c}	0.61±0.01^{b}	ND
D-G-linolenic acid (ω6)	20:3(n-6)	3.35±0.01^{a}	ND	0.63±0.01^{c}	2.88±0.14^{b}
Arachidonic acid (ω6)	20:4(n-6)	5.03±0.04^{a}	3.94±0.01^{c}	4.12±0.02^{b}	3.38±0.10^{d}
Eicosapentaenoic acid (EPA) (ω3)	20:5(n-3)	3.81±0.09^{b}	4.54±0.19^{a}	2.19±0.06^{c}	3.15±0.09^{d}
Docosahexaenoic acid (DHA) (ω3)	22:6(n-3)	9.50±0.29^{b}	5.39±0.03^{c}	9.03±0.02^{b}	10.25±0.15^{a}
∑Polyunsaturated		33.53±0.60^{a}	22.40±0.27^{d}	26.69±0.80^{c}	31.83±0.41^{b}

Data are shown as Mean (± SE). Sample size (n) =9 for each cross-type. All mean values sharing different lowercase superscripts within a row are significantly different ($P < 0.05$). RR= R♀ × R♂; CC= C♀ × C♂; CR = C♀ × R♂ and RC= R♀ × C ♂.

Table 26. Muscle fatty acid (Cumulative) composition of all cross-types

	Cross-types			
	Purebred species		Hybrids	
Fatty acids	**RR**	**CC**	**CR**	**RC**
Total FA(SFA+USFA)	98.68±3.0^{a}	92.80±10.9^{a}	91.07±5.23^{a}	93.51±4.45^{a}
∑ SFA	37.34±0.88^{c}	51.22±0.91^{a}	40.68±0.23^{b}	38.52±0.73^{bc}
∑ MUFA	27.41±0.48^{a}	19.17±0.28^{c}	23.70±0.45^{b}	23.16±0.51^{b}
∑ PUFA	33.53±0.60^{a}	22.04±0.27^{d}	26.69±0.80^{c}	31.83±0.41^{b}
∑ ω3	17.65±0.10^{a}	12.03±0.13^{d}	12.98±0.08^{c}	15.80±2.50^{b}
∑ ω6	15.88±0.09^{b}	10.37±0.10^{d}	13.71±0.01^{c}	16.03±0.15^{a}
Ratio ∑ SFA/ ∑UFA	0.62±0.02^{d}	1.23±0.03^{a}	0.81±0.01^{b}	0.70±0.02^{c}
Ratio ∑ MUFA/∑ PUFA	0.82±0.02^{b}	0.86±0.01^{ab}	0.89±0.02^{a}	0.73±0.02^{c}
Ratio (ω3/ ω6)	1.11±0.01^{ab}	1.16±0.02^{a}	0.95±0.01^{c}	0.99±0.03^{c}

Data are shown as Mean (± SE). Sample size (n) =9 for each cross-type. ANOVA followed by LSD test. All means sharing different lowercase superscripts within a row are significantly different ($P < 0.05$). RR= R♀ × R♂; CC= C♀ × C♂; CR = C♀ × R♂ and RC= R♀ × C ♂.

Discussion

Intergeneric hybridization is one of the potential genetic tools in genetic enhancement programs to generate genetic variation and improve productivity through hybrid vigor (Mallet, 2007; Litsios and Salamin, 2014). This technique involves combining the attractive traits of two different species through the exchange of genome, which leads to phenotypic and genotypic variation (Stelkens et al., 2009; Al-Harbi, 2016). The acquisition of transgressive inheritance or heterosis is the main purpose of hybridization in aquaculture. Many authors used this technique for monosex production (all female male or all male) of different fish species (El-Zaeem and Salam, 2013), while others focused on improving the reproductive performances, survival, growth, disease resistance, immunity, thermal tolerance, etc. of finfish and shellfish (Hassen et al. 2011; Reddy, 2000; Sarder et al., 2014; Um-E-Kalsoom et al., 2009; Wang et al., 2019; Ahmad et al., 2020). In the present study, hybridization between two commonly cultured cyprinid species of Pakistan, i.e., *L. rohita* and *C. catla* was performed to obtain heterosis in certain traits like growth, body shape, immunity, and thermal tolerance. It is well documented that *C. catla* is fast growing compared to *L. rohita* but carcass value, nutritive value, and immunity of *L. rohita* are better than *C. catla* (Shakir et al., 2013). Natural hybridization is quite common between *C. catla* and *L. rohita* and among many inter-specific and inter-generic hybrids from natural ecosystems have been reported by many investigators (Padhi and Mandal, 1997). The successful production of these hybrids at different hatcheries and their survival, growth, and the morphological difference is also well documented (Alikunhi et al., 1971; Reddy and Varghese, 1980; Bhowmick et al., 1981; Al-Manun and Mahmad, 2014; Sarder et al., 2014), however, limited literature revealed the comparative performance of reciprocal hybrids.

The results of interspecific hybridization depend on the genetic structure of broodstock (Bartley et al., 2001), therefore we selected the best strains of *L. rohita* and *C. catla* from Tawakkal Fish Hatchery and Farm, Muzaffargarh, and reared under the best management practices in a semi-intensive culture system. For minimizing the individual effect, milt of all *C. catla* and *L. rohita* males were pooled separately in vials and diluted with 0.9% NaCl solution and all fish were bred and reciprocal crosses were made on the same day, i.e., under similar environmental conditions. Although, higher fertilization rate (>85%) was achieved with all

cross-type, homologous crosses, i.e., both parental cross-types (CC ♀ x CC ♂ and RR ♀ x RR ♂) showed statistically similar and considerably higher ($P< 0.05$) fertilization rate than heterologous cross-types (CC ♀ × RR ♂ and RR ♀ × CC ♂) (Fig 4). Our results of statistically similar and >90% fertilization rate of both parent species (*L. rohita* and *C. catla*) were in agreement with many investigators (Chakrabarti et al., 2017; Mohapatra et al., 2018). The hatching rate also followed a similar trend and recorded significantly lower in the hybrids (Fig 4). The similar variation between fertilization and hatching rates in pure parental crosses and hybrids were also reported by various investigators in different finfish (Alikunhi and Chaudhuri, 1959; Chaudhuri, 1961; Tarnchalanukit, 1985; Morni, 2003; Raj et al., 2005; Adebayo, 2006; Moses et al., 2005; LUO et al., 2010; Akinwande et al., 2012; Tilahun et al., 2016) and shellfish (Lyu and Allen 1999; Rahman et al., 2000, 2001, 2004 & 2005; LUO et al., 2010). The higher fertilization and hatching rates of homologous crosses may be related to easily attachment of sperm to conspecific ova than to heterospecific ones (Leighton and Lewis 1982; Shah et al., 2008). Previous investigations reported the role of membrane proteins in gamete recognition or sperm-egg interactions and suggested that the low fertilization rate of heterologous crosses could be attributed to the surface glycoprotein of the eggs, which might be different in two distinct species (Palumbi, 1994; Biermann, 1998). Besides genetic factors, many non-genetic factors like water temperature, pH, alkalinity, salinity, and hardness also contribute significantly to the success of hybridization (Gao et al., 2011; Rahman et al., 2012). However, in this study, all the breeding experiments were conducted on the same day in similar environmental conditions.

Here, we also observed statistically similar but slightly better fertilization rate and hatching rate in the hybrids of *L. rohita* eggs (R ♀ × C ♂) compared to its reciprocal hybrids i.e., *C. catla* eggs (C ♀ × R ♂). This may be related to the quality of eggs, as in comparison to *C. mrigal* and *C. catla*, the egg size and diameter of the micropyle canal of *L. rohita* egg is somewhat larger than the size of the sperm head (Gopalakrishnan et al., 2002; Khan et al., 2005). Several investigators reported variation in the size, structure, and development of micropyle in various teleost and related this to fertilization rate (Riehl, 1980; Guraya, 1986; Yamamoto and Kobayashi, 1992, Chen et al., 1999). Somewhat higher fertilization rate of rohu in contrast to *C. catla* has already been reported by many authors (Singh et al., 2006; Sahoo et al., 2017; Mohapatra et al., 2018). Like our observation, Tilahun et al. (2016) also observed a slight

difference in fertilization and hatching rate in the cross of batrachus eggs with *gariepinus* sperm (*batrachus* ♀ × *gariepinus* ♂) in contrast to its reciprocal hybrids (*Gariepinus* ♀ × *batrachus* ♂). However, Legendere et al. (1992) observed significant differences in hatching rates of *Clarias gariepinus* and *Heterobranchus longifilis* and their reciprocal hybrids and suggested that hatching rates are mostly determined by egg quality or species. Furthermore, Li et al. (2019), did not observe any significant difference in the fertilization rate and hatching rate of parent species and hybrid of female Bleeker's yellowtail and male topmouth culter, while Shivaramu et al. (2019), reported significantly higher fertilization and hatching rates in Siberian sturgeon (♀) × Russian sturgeon (♂) hybrid group than parental species. This variation in results may be related to species differences or environmental conditions.

In aquaculture, reproductive performance is the complementary indicator, while growth rate, disease resistance, survival (%), nutritive value are probably the most driving and desirable indicators (Bartley et al. 2001). Here we also evaluated the growth performance of both hybrids in contrast to parental species. For true ranking and evaluation of genetic vigor, we conducted experiments on different development stages of fish in both control and semi-intensive culture system. In early developmental stages, numerous environmental factors like age, maternal effects, mortality, temperature, stocking density and water quality can affect the phenotype of an individual and need to be controlled or corrected (Dunham, 2011). Here, for avoiding or minimizing environmental influence, we used the best strain (fast growing diseases resistant) of similar age and size broodstock of each species, which were reared for 3 months in similar environmental conditions and on the same feed and feeding strategy. Moreover, we selected the post-larvae (after yolk sac absorption, i.e., 4 days old spawn) of all cross-types and reared them separately both in earthen nursery ponds (in semi-intensive culture system) and fiberglass troughs in controlled conditions. Further, the environmental effect was minimized by the rearing of each cross-type in a replicate of three in similar size of ponds /trough, for 3 months (mid-June to mid-August) at the same stocking density in almost similar environmental conditions (DO, temperature, pH, alkalinity, hardness, etc.) while age effect which could mask the genetic effects (Dunham, 2011) was controlled by conducting a breeding experiment on the same day and using post-larvae of all cross-types which were hatched on the same day.

During early development, survival % generally depends on environmental factors including quality of eggs (maternal effect), feed, presence of predators, and water quality (Hepher, 1988; Zak et al., 2007). Here, all cross-types were also reared in triplicate in similar environmental conditions, both in control and semi-intensive culture system. In nursery ponds, pond fertility and water quality were maintained by daily addition of fresh water at dawn and dusk and fortnightly checking the fertility of pond by Secchi disk and addition and restriction of fertilizer accordingly. All ponds were in the same vicinity, therefore did not show any significant difference in water quality parameters. Moreover, maternal effect (environmental) was minimizing by rearing broodstock of both species in a communal pond on the same feed and feeding strategy. Here, the observed significant difference in survival % among postlarvae and fingerlings of all cross-types after 90 days of rearing under control condition may indicate the involvement of genetic factor. The results indicated the lowest survival rate of *CC* (*C. catla*) and the highest survival of RR (*L. rohita*) during both life stages and it is in accord with literature which showed a low survival rate of *C. catla* as compared to *L. rohita*, *Cirrhinus mrigala*, and *Cirrhinus cirrhosus* (FAO, 2009; 2014).

Literature indicated considerable variation in the survival rate of *C. catla* and *L. rohita* within species and between species and revealed the influence of many factors like life stage, size, stocking density, stocking ratio and culture system (monoculture or polyculture), culture management (controlled or semi-intensive), species, feeding regimen, and duration of grow-out on the survival % of both species. For instance, 91% survival of *C. catla* was observed by Basavaraju et al. (1995) after 156 days of rearing of fry (BW, 1.57 g) in a concrete tank. However, a lower survival rate (44-47%) of fingerlings (BW, ≈ 40g) of the same species in contrast to *L. rohita* (64-85%) fingerlings (BW, ≈ 21g) was reported by Sarder et al. (2014) after rearing *C. catla* along with *L. rohita and C. mrigala* and their hybrids in an earthen pond under a polyculture system over two years. Al-Mamun and Mahmud, (2014) also reported the lower survival rate of *C. catla* (43.75%) in contrast to rohu (63.89%) after rearing *C. catla* (BW, ≈291g) and *L. rohu* (BW, ≈230g) along with *C. mrigala* and their hybrids at different stocking ratios for about nine months in an earthen pond under semi-intensive culture system. Similarly, Azim et al. (2014) also reported a lower survival rate of *C. catla* (45.56 - 86.85%) in contrast to *L. rohita* (66.37% to 95.45%) while rearing fingerlings of these species

in various combinations in earthen ponds for about 70 days. Our results also indicated significant variation in the survival rate % within species and between species at different life stages of fish, i.e., the higher mortality rate (*C. catla*, > 50%; *L. rohita*, <25%) during early rearing as compared to the rearing of fingerlings of both species (*L. rohita* < 8%, *C. catla* >20%). The observed > 60% survival rate of *L. rohita* during early rearing in control conditions was in accordance with results reported by other investigators (Ibrar et al., 2017; Amir et al., 2018). Similarly, the lower mortality rate of *L. rohita* about 10% during the rearing of the fingerlings (BW, ≈ 20g) in a semi-intensive culture system was also reported by Amir et al. (2018).

The observed survival % of both F1 hybrids under both rearing conditions and at different life stages in the present study was intermediate as compared to parental species, may be due to disease resistance, stress tolerance and immune response being greater than *C. catla* and lower than *L. rohita* (Table 4). Like our results, Sarder et al. (2014) also reported the intermediate survival rate of *L. rohita* × *C. catla* hybrid (BW, 15g) while rearing this hybrid in earthen ponds for 2 years with fingerlings of parental species (*L. rohita* and *C. catla*), *C. mrigala*, and hybrids of *C. mrigala* × *C. catla* and *L. rohita* × *C. mrigala* Moreover, Al Mamun and Mahmud also reported an intermediate survival rate of *L. rohita* × *C. catla* as compared to parental species after conducting series of rearing experiments by using different stocking ratios of *C. catla, L. rohita, C. mrigala*, and their hybrids in an earthen pond under semi-intensive culture system for 9 months. Here we also observed a significant difference ($P<0.05$) in the survival rate of both hybrids, i.e., RC hybrids showed a better survival rate as compared to CR hybrids. No literature is available to compare the results of survival % of reciprocal hybrids of *C. catla* and *L. rohita*. However, like our results reciprocal hybrids of *C. gariepinus* and *H. bidorsalis* showed a significant difference in survival rate, i.e., 78% for *H. bidorsalis* ♀ × *C. gariepinus* ♂ in contrast to 61 % for *C. gariepinus*♀× *H. bidorsalis* ♂ during early rearing of fry for 14 days (Owodeinde et al., 2013). Moreover, unlike over results reciprocal interspecific hybrids of *C. gariepinus* × *C. anguillaris* showed an almost similar survival rate during early rearing. Furthermore, our results of significantly decreased in the mortality rate of all cross-types (purebred species and reciprocal hybrids) with an increase in body weight were also in accord to literature that reveals an increase in the survival rate of fish with an increase in body length and body weight of fish (Pepin, 2016; Stige et al., 2019).

In aquaculture, survival and growth are the most desirable traits of any culturable species. These traits are directly related to net production per unit area (Al-Mamum and Mahmud, 2014) and affected by many factors including, water quality parameters, soil quality, species, genetic make-up and developmental stage of species, stocking density, infectious diseases, etc. In this study, for true genetic ranking of all cross-types based on growth performance, we conducted growth evaluating experiments of all-cross-types at different life stages, both in controlled and semi-intensive culture system (Tables 4,5,6,7,8). Results of this study except under suboptimum culture condition (during winter) indicated a similar growth pattern of all cross-types, i.e., a faster growth rate of CC (*C. catla*) cross-type followed by hybrids (CR and RC), while RR (*L. rohita*) showed the lowest growth rate. Under controlled conditions, two sets of experiments, each for 90 days, one for early rearing of swim-up fry in fiberglass trough (capacity, 150 L) and the other for the rearing of fingerling (BW:- range 25-31g) in communal fiberglass tank were conducted. During rearing, optimum water temperature (27˚C) and DO level (5.5- 6mg/L) were maintained by using water heaters and aeration system while other parameters like pH, total ammonia, hardness and, alkalinity were also found in range optimum range for the rearing of Indian major carps. Moreover, the water quality of each trough/tank was maintained by removing uneaten feed and fecal matter through siphoning and exchanged 20% of tank water with fresh water daily or the once every two days.

For evaluating growth performance of all cross-types in the semi-intensive culture system, efforts were made to culture all groups of fish under similar rearing environment by selecting nursery ponds, grow-out ponds, and communal pond which were in the same vicinity and adjacent to each other and rearing of all groups was conducted in triplicate. All ponds were fertilized at the same rate with the same organic and inorganic fertilizers and also provided supplementary feed at the same ratio. During rearing, although, water quality parameters showed variation but did not show any noticeable change between ponds. Moreover, water temperature during the early rearing and rearing of fingerlings in communal ponds showed increasing trends but it remained within a range suitable for the rearing of Indian major carp.

Pakistan has a continental type of climate, i.e., annual variation in temperature. Water temperature in most of the areas of Punjab starts to decline in September and reaches a minimum

value in January and then gradually rises and showed peak values during June-July. Therefore, during September, fingerlings of all cross-types were shifted from nursery ponds to grow-out ponds at a stocking density of 5000 fingerlings/ ha as suggested by Gjerde et al., (2019) and reared there for about 250 days. During the rearing period, extreme weather conditions, i.e., thunderstorms, rainy/cloudy season, and extreme daily variation in water temperature were observed and we adjusted the feeding rate accordingly. It is well documented that fish feeding activity is reduced during the colder season or when the temperature is < 20˚C (NAERLS, 2002). Therefore, feeding was restricted during rainy or cloudy weather and limited the feeding rate at low temperature because, in this condition, most of the fish do not feed on floating pellets and depend on naturally occurring feeds in the pond waters (Ramakrishna, 2013). The same feeding strategy was adapted for all cross-types. After this rearing period, we observed a very different trend in the growth of all cross-types i.e., rohu showed the highest %WG followed by RC hybrid, while *C. catla* showed the lowest %WG. It may be due to rearing temperature. During the rearing period, water temperature remained below the optimum temperature required for the growth of Indian major carps. It is well documented that thermal tolerance is species-specific and *C. catla* (surface feeder fish) is the least temperature tolerant species than *L. rohita*, which feeds at the column or middle layer of the pond due to which they have more tendency to adapt to adverse conditions (Das et al., 2004). It seems that during this rearing period, *C. catla* used most of the energy for tolerating thermal stress instead of growth.

In this study, all cross-types were reared almost in similar environmental conditions; therefore the observed difference in growth rate may be related to their genetic make-up. Among Indian major carps, *C. catla* is the fastest-growing species (FAO, 2013; Al Mamun and Mahmud, 2014) and we also observed this trend both in the controlled and semi-intensive culture system. Postlarvae and fingerlings of CC-cross-type showed significantly higher %WG and daily weight (ADG) in comparison to *L. rohita*. Although, during the early rearing in the controlled condition the ADG values of both species did not show any significant difference, the difference was obvious in nursery ponds and during the rearing of fingerlings. Our results of higher weight gain and SGR of *C. catla* as compared to *L. rohita* during the early rearing of these species were in accordance with Nandeesha et al. (2001). Moreover, other investigators reported a significantly higher weight gain (>34%), and SGR of *C. catla* as compared to *L. rohita* after six

months (Al-Mamun and Mahmud, 2014) and two years (Sarder et al., 2014) of rearing of fingerlings in polyculture system.

In the present study both hybrids (CR and RC) showed intermediate growth performance, i.e., higher %WG compared to *L. rohita* but less than *C. catla*. The literature revealed comparatively similar growth of hybrids and parental species in the initial developmental stage compared to the grow-out stage (FAO, 2012). However, we observed a significant difference in FBW, WG, %WG and SGR% of both hybrids with parental species after 90 days of rearing of post-larvae in both controlled and semi-intensive culture systems. Both hybrids showed significantly higher values of growth indices than RR cross-type but comparatively fewer values compared to CC cross-type. This discrepancy in results may be related to the rearing period; we observed results after 90 days of rearing of post-larvae, whereas most investigators reported early growth rate after 15-30 days of rearing. Additionally, during the early life stage, both hybrids showed low parent heterosis, and only the CR hybrid had shown favorable mid-parent heterosis for body weight and SGR.

Literature revealed the variable growth rate of both hybrids in the grow-out stage. For instance, some investigators reported that the growth performance of the hybrids produced from *L. rohita* ♀ × *C. catla* ♂ cross is better than slower-growing parent *L. rohita* (Varghese and Shantharam, 1979; Keshavanath et al., 1980; Konda Reddy and Varghese, 1980; Maheshwari et al., 1990; Jana, 1993), while others reported no significant difference in the growth rate of this hybrid (RC) and *C. catla* (Chaudhuri, 1973). Konda Reddy and Varghese (1980b) reared both hybrids (RC and CR) along with the parent species and reported that RC hybrid grows slightly faster than rohu (RR) but very much slower than catla (CC). However, like our study, Keshavanath et al**.** (1980) also assessed the growth performance of both hybrids (RC and CR) along with their parental species (RR and CC) under solitary (independent) and communal rearing conditions but unlike our results reported insignificant difference in the growth of CR and RC hybrids. Although, both RC and CR hybrids did not show the most pronounced difference in growth-related indices (WG, %WG, SGR, and ADG) while rearing in solitary at a suboptimum temperature under a semi-intensive culture system the difference was obvious in communal rearing conditions (both controlled and semi-intensive) and CR hybrid

showed better growth performance compared to RC hybrid. Our results of better growth performance of both hybrids compared to *L. rohita* and inferior to *C.catla* while reared in solitary are in accord to Keshavanath et al**.** (1980) but in communal rearing, results are variable, we observed a similar trend, i.e., growth performance of hybrids better than *L. rohita* and lower to *C. catla* but Keshavanath et al. (1980) did not observe any significant difference in growth-related indices of *L. rohita* and hybrids.

Some other scientists also determined the growth of CR and RC hybrids in the grow-out stage of different water bodies. For instance, Maheshwari et al. (1990) stocked RC hybrid in 3 different water bodies (large and small reservoirs, ponds) and reported inferior growth of hybrid (< 10-20%) compared to the male parent (*C. catla*) while 30-125% better compared to the female parent (*L. rohita*) in the pond culture system. In a small reservoir, although in the first year, they did not observe any significant difference among the growth of catla and hybrid, nevertheless in the second and third year the difference became obvious and hybrids showed 20% and 32% respectively less weight compared to *C. catla*. During three years rearing period hybrids showed 53-128% higher growth over rohu. However, over 2 years of rearing in a polyculture system consisting of Catla, rohu, and mrigal and their hybrids, Sarder et al. (2014) reported 8-24% higher weight of hybrids compared to rohu while 12-36% less weight compared to Catla. Moreover, over 9 months of rearing of rohu, catla, mrigal, and their hybrids, Al-Mamun and Mahmud (2014), reported a higher % weight gain of CR hybrid compared to both parents i.e., 17.6% higher than catla and about 77% higher than rohu.

Overall results of this study concerning growth traits indicated the intermediate growth of both hybrids in comparison to both parents species. Although both F1 CR and RC hybrids had shown low parent heterosis for growth-related parameters (Fig 8,9,10), yet F1 CR hybrids showed relatively higher LHP and positive MPH than F1 RC hybrid. It seems a faster-growing parent (*C. catla*) transmitting this trait (growth potential) to hybrid. Like our results, Kumolu-Johnson et al. (2011) also reported the more weight gain and higher specific growth rates of the reciprocal hybrid of female *C. gariepinus* (CG)× male *H. bidorsalis* (HB) than the hybrid HB × CG and suggested the maternal influence on the growth-related indices of fish. Based on growth performance, the genetic raking of all-cross-type (CC> CR>RC >RR) also confirmed the

stronger effect of females in crosses as compared to males. Many investigators reported similar maternal effect in a wide variety of species (Ibanez-Escriche et al., 2014; Liu et al., 2016).

For the validation of results of growth performance of all cross-types, we further extend our study and determine the mRNA levels of growth-related genes, MyoD, Myogenin, and IGF-1 in liver and muscle of all cross-types after 90 days of communal rearing of fingerlings in a semi-intensive culture system. Data collected from adult and juvenile fish indicated that the rate of growth in teleost is a species-specific phenomenon and can be affected by external factors like temperature, nutrient availability, water quality parameters, culture conditions, etc. (Steinbacher et al., 2007). Generally, growth in teleost involves the hypertrophy and recruitment of muscle fibers (Johnston, 1999). Many investigators indicated the persistence of both hyperplasia and hypertrophy in fast-growing fish (Steinbacher et al., 2006), while the limited rate of hyperplasia and the dominance of hypertrophy in slow-growing fish (Weatherley et al., 1988; Veggetti et al., 1993).

Literature reveals that recruitment of new muscle fibers in fish occurs throughout their life (Rowlerson and Veggetti, 2001; Sengupta et al., 2014) and the process is under the control of myogenic regulatory factors (MRFs). In teleost, these factors comprise MyoD, Myf5, myogenin, and MRF4 can convert a variety of non-muscle cells into myotubes and myoblasts (Emerson, 1990; Weintraub, 1993). The present study indicated a significant difference in the expression of MyoD and myogenin among cross-types. The mRNA levels of both MyoD and Myogenin followed the same pattern as observed in growth, i.e., higher expression in CC followed by CR and RC cross-type while lowest in RR cross-type. Both genes showed tissue-specific expression i.e., higher expression in muscle as compared to liver. These results are in accord with literature that showed the higher expression of these genes in muscle and indicated their role in myogenesis (Rescan, et al., 194; Kim, et al., 2004)

Insulin-like growth factor-I (IGF-I) is an important hormone, mostly secreted by the liver and transported to other tissues. It plays an important role in the somatic growth of teleost via growth hormone action (Moriyama et al., 2000). Growth hormone and IGF-I are the two most important genes affecting the growth of fish (Triantaphyllopoulos et al., 2019). Many

investigators reported the role of IGF-I in the growth of channel catfish (Silverstein et al., 2000; Peterson et al., 2004). Besides affecting somatic growth, IGF-1 also plays role in tissue repair, osmoregulation and metabolism of proteins, carbohydrates, and lipids and affect the immune system by enhancing cell proliferation, lymphopoiesis, cell survival, and granulopoiesis (Clark et al., 1993: Kooijman et al., 1996; McCormick, 1996). Many scientists reported the tissue-specific expression, i.e., highest in the liver compared to other tissues (Power et al., 2000; Ayson et al., 2007; Dar et al., 2018). Here we also observed higher expression of this gene in the muscle of all cross-types compared to the liver. Moreover, the IGF-1 mRNA level showed a similar pattern among all cross-types as shown by MyoD and Myogenin genes, i.e., CC cross-type showed the highest expression followed by CR cross-type while RR cross-type showed the lowest expression.

In aquaculture, accurate identification of species including their hybrids is important for a better understanding of biodiversity issues, assessment of production, and sustainable development of aquaculture. Before 1960, morphological study was in practice for the identification of species. Nowadays, still it is frequently used for the evaluation of genetic diversity, identification and classification of aquatic animals, and population differentiation. Worldwide, it is in practice for segregating fish species (Betancur et al., 2017; Guan et al., 2017; Ukenye et al., 2019), Morphometry generally based on body shape, number, size, color, and pattern of scales, fins number and position, fin rays number and form, as well as measurements of several body parts (Telechea, 2009). Usually, individuals would be considered hybrids when their meristic and morphometric measurements values were intermediate to values of two parental species (Hubbs and Kuronuma, 1942; Campton, 1987; Teletchea, 2009). However, hybrids can express differently among traits, leading to violation of the intermediate assumption. Many factors like environmental factors (Binning and Chapman, 2010), diets, developmental stages (Biswas and, Shah 2009; Dwivedi and Dubey, 2013), and the genetic origin of populations (Janhunen et al., 2009; Biswas and Shah, 2009) affect the morphological characteristics. To avoid environmental influence, fingerlings from communal ponds were selected for the morphological identification of all cross-types.

Our results indicated a difference in body shape, size, color, scales shape and size, mouth gap, eyes position, and head size of *C. catla* and *L. rohita*. The body shape of *L. rohita* was fusiform with blackish color at the dorsal side and silvery below and the sides compared to *C. catla* which had a short, deep, and somewhat laterally compressed body that was greyish at the dorsal side and silvery-white at the ventral region. The mouth of *L. rohita* was sub-terminal with square or somewhat circular mouth gap/opening compared to *C. catla*, which had a broader head, wide, upturned mouth with circular large opening/gap with protruding lower jaw. The position of eyes in *L. rohita* was dorsolateral, not noticeable from outside of the head while catla had larger eyes with a red spot on the lower part of the eyeball. The lower lip of *L. rohita* was folded and fringed while the upper lip extending and covered the lower lip while in catla the upper lip was thin while the lower lip was infringed and thick/folded. Pelvic fins of *L. rohita* were red in contrast to catla that was darkish in color. Although both species had cycloid scales, yet *L. rohita* scales were diamond shape while *C. catla* scales were larger in shape. Our results are in accord with most of the investigators (Desai and Rao, 1970; Chaudhuri, 1974; Natarejan and Mishra, 1976; Reddy and Varghese, 1980; Bhowmick et al., 1981; Sarder et al., 2011).

The external morphological features indicated that both hybrids (RC and CR hybrids) were intermediate in most of the traits, while some traits resemble their mother species and both hybrids showed more resemblance towards the female. For instance, like catla, the mouth of the CR hybrid was terminal with a circular opening, curved lateral line, and black color pectoral, ventral and anal fins. However, other features were intermediate, e.g., head smaller than the CC cross-type but larger than RR cross-type and had moderately wide mouth opening and intermediate size cycloid scales, etc. Similarly, rohu RC hybrids had folded lower lip, eyes without red spot, curved lateral line, and red color pelvic fins. However, their head size was intermediate (larger than RR cross-type but smaller than CC cross-type) and their body color at dorsal region was greyish like CC and at ventral region was white like RR. Many investigators who worked on morphometric and meristic characters of Indian major carps (catla, rohu, and mrigala) also observed similar features of hybrids (Desai and Rao, 1970; Chaudhuri, 1974; Natarejan and Mishra, 1976; Reddy and Varghese, 1980; Bhowmick et al., 1981) and conclude that most taxonomic characters were intermediate between the parent species. Like our

observation, Desai and Rao, (1970) reported feebly fringed lower lip and terminal mouth of CR hybrid, while Tripathi et al. (1974) reported extensively thick mouth with striated lips. Similarly, Natarajan et al. (1976) reported a terminal mouth without barbels of CR hybrid. However, except for some minor characteristics, Reddy and Varghese, (1980) did not observe any difference between CR and RC.

For accurate identification of hybrids and parental species, we further calculated some morphometric ratios, and counted the fin rays of all cross-types. In morphometric ratio, the significant difference ($P>0.05$) among cross-types was found only in TL ratio with HD, ED, PrePL and, PreAL. Similarly, out of 18 meristic counts, only 9 counts showed significant differences ($P<0.05$ among cross types. Both CR and RC hybrids showed a significantly higher number of scales above and below the lateral line and a total number of pectoral-fin rays while intermediate numbers of branched and un-branched caudal-fin rays. Moreover, some ratios showed maternal influence like the total number of dorsal fin rays. Overall results indicated that most morphometric characters of both hybrids are in accord with literature and although both hybrids were intermediate in appearance but showed more maternal resemblance (Bhowmick et al., 1981; Al-Mamun and Mahmud, 2014).

Many scientists reported the maternal inheritance of meristic trait however, our results showed variable results and did not follow intermediacy or maternal influence. The observed variation may be explained by their genetic potential or environmental influence (Al Mamun and Mahmud, 2014). The impact of rearing environment or environmental fluctuations on the morphometric traits of fish is well documented (Allendorf and Phelps, 1988; Swain et al., 1991; Wimberger, 1992). According to them, fish are more flexible and adopted the changing environment by essential morphometric changes both within and between populations. Like our results, Šimková et al. (2015) also observed variable results. They reported maternal inheritance of some morphometric characters into the hybrid of common carp × gibel carp into carp, while the paternal inheritance of some meristic traits and suggested that body morphology of hybrids could be inherited from paternal or maternal species in an equal or unequal manner. Conversely, some investigators reported maternal or paternal predominance. For instance, Park et al. (2006) reported the maternal inheritance of some morphometric traits in the hybrid of mud loach

(*Misgurnus mizolepis*) and cyprinid loach (*Misgurnus anguillicaudatus*), while Dunham et al.(1982) reported more resemblance of blue and white channel catfish hybrid to paternal species

The morphological identification of fish species based on morphometry and the meristic count is sometimes misleading (Zhu et al., 2013). To avoid ambiguity and identified the hybrids and parental species more accurately, a molecular approach, i.e., DNA barcoding by mitochondrial COX 1 gene was undertaken. It is well known that transcription of mtDNA encoded genes varies in the population, thus these genes can be used to illustrate variances among populations or species and to examine deep divergence (Suneetha et al., 2000; Apostolidis et al., 2008; Wu et al., 2010; He et al., 2011; Mandal et al., 2012). Nowadays, the CO1 gene along with other mitochondrial genes is used to identify interspecific and intraspecific hybrids and to determine their phylogenetic relationship with parental species (Akhtar and Ali, 2016; Qu et al., 2018). Moreover, the CO1 gene estimated the nucleotide base difference among species, genera, and families and accurate discrimination of unidentified species (Dawany et al**.,** 2007).

Here amplified barcode region of the mitochondrial DNA of all cross-types was sequenced and a complete barcode fragment of 670bp (RR cross-type), 672bp (CC cross-type), 644bp (CR cross-type), and 659bp (RC cross-type) was obtained. The result indicated higher levels of transitional substitution than transversional substitution, which is in accord with results reported by Ayesha et al. (2019). In RR cross-type, out of 670bp nucleotide length (excluding sites with alignment gaps), 97.9% (656) sites were monomorphic while 2.1% (14) sites were polymorphic. Out of 14 variables, 9 were singleton and 5 were parsimony informative. However, Ayesha et al. (2019) reported somewhat different results of CO1 analysis sequence analysis of rohu, i.e., 89% in variable or constant characters and about 11% variable polymorphic characters with 28 singletons and 65 parsimony informative sites. Here, the observed total number of haplotypes (4) and haplotypes diversity (Hd, 0.455) were comparatively less than 10 and 0.933 respectively reported by Ayesha et al. (2019). However, the sequence analysis of the nucleotide composition of the CO1 gene was in accord with the literature. The observed small intraspecific distance (0.003) compared to 0.026 reported by Ayesha et al. (2019) indicated small intraspecific variation which may be due to sampling size.

CO1 sequence analysis of CC cross-type (*C. catla*) indicated the mean nucleotide length of 672bp excluding the sites with alignment gaps or missing data. Out of 672 sites, 655 sites (97.5%) were found to be constant (monomorphic), while 17 sites (2.5%) were variable (polymorphic) with 11 singletons and 6 parsimony informative sites. The total number of haplotypes, haplotypes diversity and nucleotide diversity were somewhat higher than the RR cross-type but less than reported for *C. catla* (Ayesha et al., 2019). The observed nucleotide composition of CO1 gene sequence in CC cross-type was similar to RR cross-type but slightly variable compared to values reported in the literature. The intraspecific distance was less than the RR cross-type indicating the least intraspecific variation in the samples of CC cross-type.

CO1 sequence analysis of CR cross-type (hybrid) indicated an average nucleotide length of 644 bp excluding the sites with gaps or missing data. The 644bp consisted of a higher number of invariable or monomorphic sites (633 or 98.2%) and less number of variable polymorphic sites (11 or 1.71%) compared to RR and CC cross-types. Moreover, CR cross-type also showed less number of singletons sites in CR hybrids compared to RR and CC cross-types while parsimony informative sites were similar to RR cross-type and less than CC cross-type. The total number of haplotypes in CR-cross-type was 6 compared to 4 and 5 observed in RR and CC cross-types. CR cross-type also showed higher haplotypes diversity while lower nucleotide diversity compared to parental species. The observed nucleotide composition of COI gene sequence in CR cross-type also showed variation compared to parental species e.g. lower contents of cytosine and somewhat higher contents of Guanine with comparatively higher A+T and lower G+C contents

Like COI sequence of CR cross-type, COI sequence of RC hybrid also showed variation compared to parental species. The mean nucleotide length of 659 bp excluding the gaps consisted of 650 (98.64%) invariable (monomorphic), and only 9 (1.37%) variable (polymorphic) sites. Here the %age variable sites including parsimony informative sites were less than observed in other cross-types. Like RR cross-type, the number of haplotypes was 4 but haplotype diversity was greater than RR and CC cross types, and less than CR cross-type. The observed nucleotide composition of CO1 gene sequence in RC cross-type also showed variation compared to other cross-types (parental species and CR cross-type). Although like CR hybrid the

nitrogenous bases in nucleotide composition followed the same raking, i.e., T>A>C>G but the % contents were variable, i.e., %content of G and % G+ C was highest in RC hybrids compared to all cross-types while % T and % A were slightly less than CR hybrid. No literature is available on the COI sequence analysis of rohu-catla hybrids (RC and CR) for comparison of our results.

The present study also indicated that all cross-types showed an obvious anti-G bias, more prominent in parent species (RR and CC cross-types) compared to hybrids (CR and RC). This is in accord with literature indicating the more common appearance of anti-G bias in a teleost (Zhu et al., 2013; Akhtar et al., 2017). For instance, many investigators reported such G-deficient nucleotide patterns in different fish species like rohu and catla (Ayesha et al., 2019), *Carassioides acuminatus* (Zhu et al., 2017). Moreover, all cross-types showed a high rate of transitional substitution (10-13.6%) compared to transversional substitution (4.72-6.23%)

Furthermore, the constructed Neighbour Joining (NJ) tree (K2P) based on barcode sequencing with a region of approximately 680bp for all samples of four cross-types, i.e., (RR, CC, CR, and RC hybrids) indicated two clades, i.e., representing two separate species. The analyses of the histogram of genetic and ranked distances revealed a clear gap between conspecific and congeneric species. Each hybrid is closely related to its female parent and showed an identical phylogenetic relationship with the mother species. For instance, CR hybrids showed the minimum distance with *C. catla* while RC hybrid showed the minimum distance with *L. rohita***.** Based on the K2P model, the interspecific divergence between parent species and hybrid ranges from 0.003-0.014 (Table 19). The maximum genetic distance was noticed between *C. catla* and RC hybrid and the lowest distance was observed between *L. rohita* and RC hybrid**.** It appears that, although all the mitogenomes of the hybrids were consistent with maternal inheritance, yet a certain level of variation was detected in the mitogenomes. The number of variable sites was found to be related to the phylogenetic relationship of the parents.

Besides affecting the growth and general morphology of fish, hybridization can bring out changes in the physiology, blood chemistry, disease resistance and immunity of fish. Many investigators studied the hematological parameters of hybrids to parental species for the evaluation of genetic flexibility of fish against pathogens (Diyaware et al., 2013; Šimková et al.,

2015; Zhang, 2019). and confirmed the impact of hybridization on the hematological parameters of fish. Hematological parameters reflect the health status and general physiology of fish (Fazio, 2019; Ahmed et al., 2020) and are affected by many endogenous (sex, species, age, etc.) and exogenous factors (stress, nutrition, husbandry practices, pollutants, feeding habits, etc.). According to many investigators, hematological studies could be helpful in the selection of fish or other animals that are genetically tolerant to stress and certain diseases (Mmereole, 2008; Isaac, 2013; Ahmad et al., 2020).

Here we also studied the hematological indices of all cross-types for the assessment of their health status and potential to cope with stress and disease and found a significant difference. To avoid the environmental influence, the hematological profile of *C. catla, L. rohita* and their reciprocal hybrids (CR and RC hybrids) were investigated under the same environmental condition, i.e., fish of all cross-types of the same age were first reared in the communal pond for 90 days and then acclimatized for 2 weeks under laboratory conditions before taking blood. All blood parameters of fish recorded in the current study were within the ranges for healthy fish, however, showed significant difference among species and their intergeneric hybrids. Both F1 hybrids (RC followed by CR hybrid) showed the highest value of RBCs, WBC MCH, MCHC, HB, and Hct% as compared to parental species. It seems hybrids were more tolerant to stressor compared to both parental species. Several hematological indices like Hb, Ht, RBCs indicate the oxygen-carrying capacity and secondary responses of animals to irritants (Shah and Altindag, 2004; Adeyemo et al., 2008). Moreover, among hematological parameters, WBCs are related to the defense mechanism or immunity of fish and their higher number in both hybrids may indicate their comparatively higher stress resistance. Several exogenous and endogenous factors like fish species, genotype, age, infection to pathogens, and nutritional differences may change the number of WBC (Magnadottir, 2006; Romano et al., 2017; Parrino et al., 2018). Overall, in both hybrids, higher values of hematological parameters indicated their better potential to cope with stress compared to both parental species in a similar rearing environment. Like our results, Diyaware et al. (2013) also observed a higher level of RBCs in *C. anguillaris* × *H. bidorsalis* hybrid, while, in contrast to our observation, reported lower levels of other parameters compared to both parental species. Conversely, Šimková et al. (2015) reported an

intermediate level of WBCs in the hybrid of Cyprinus *carpio* × *Carassius* compared to parental species.

Infectious diseases are major constrain in the sustainable development of aquaculture. A disease not only affects fish production through high mortality and impaired growth but also causes financial losses and negatively impacts the livelihood of farmers (Houston, 2017). With the expansion of aquaculture and the introduction of intensive and super-intensive culture systems, the demand for genetically disease-resistant stock has been increased and disease resistance becomes a highly targeted trait for aquaculture. Many investigators have used interspecific hybridization to improve disease resistance and immunity of the stock (Rahman et al., 2013; Šimková et al., 2015; Zhang et al., 2019).

In fish, the immune system protects against disease by recognizing and eliminating disease-causing organisms. The innate immunity comprising of physical barriers (epithelial/ mucosal membrane surfaces), and nonspecific noncellular (humoral) and cellular components provide the first line of defense and are very important in disease resistance. Innate immunity is a heritable trait (Carroll and Janeway 1999; Du Pasquier 2004; Alvarez-Pellitero 2008) that is modulated by several inherent (sex, age, maternal effects) and environmental factors (temperature, stress, stocking density, etc.) and showed great diversity in the activity of immunological indices among species and even between individuals (Magnadottir, 2010). Many workers reported a significant correlation between innate immunity parameters and disease resistance of fish. For instance, Balfry et al. (1994) reported a relation between disease resistance to Vibrio species and plasma lysozyme activity, differential WBCs count, and phagocyte respiratory burst activity in tilapia and Coho salmon. Other scientists observed relation of serum lysozyme activity with *Vibrio salmonicida* infections and resistance to *Aeromonas salmonicida*, and *Renibacterium salmoninarum* in salmonids (Lund et al., 1995), serum bactericidal activity of rainbow trout with furunculosis resistance (Hollebecq et al., 1995), *Edwardsiella tarda* infection with serum lysozyme activity and mRNA level of immune-related genes (C-type lysozymes, G-type lysozymes, interleukin-1β, complement component C3, β2-microglobulin, inducible nitric oxide synthase and tumor necrosis factor-alpha (TNF-α) in *L. rohita* (Mohanty and Sahoo, 2010)

In the present study, we also evaluated the disease resistance and innate immunity of hybrids and parental species of the same age groups by designing and conducting an experiment in controlled conditions. Fish of all cross-types were challenged with *A. hydrophila* a commonly used pathogen to elucidate immune mechanisms in warm water fish (El-Houseiny et al., 2021). For comparative evaluation of the potential to cope with the pathogen exposure, blood samples of all cross-types (CC, RR, CR, and RC) were samples after 24 hrs infection and analyzed nonspecific (lysozyme activity, phagocytic activity, phagocytic index, and respiratory burst activity) and specific (IgM level) immunity parameters. Moreover, temporal and spatial expression of immune-related genes (lysozyme C, lysozyme G, and TNF-α) in muscle and liver samples were also analyzed by collecting samples at different time intervals, i.e., at 0, 6, 12, 24, 48, and 168 hrs post-infection.

Lysozyme is an important component of nonspecific defense mechanisms that has strong antibacterial activity (Grinde et al., 1988; Zarkadis, et al., 2001; Sahoo, 2004 & 2006; Saurabh and Sahoo 2008). The activity of lysozyme changed in the state of disease and response to bacterial invasion (Bunlipatanon and U-Taynapun, 2017; Bilodeau-Bourgeois and Bosworth BGPeterson, 2008). It directly attacks the cell wall of both gram-positive and gram-negative bacteria and hydrolyzes the peptidoglycan layer by splitting glycosidic bonds (Yano, 1996). In fish, lysozyme activity has been found in many tissues and secretions (Alexander and Ingram, 1992). Here like Mohanty and Sahoo (2010), we used blood serum for the analysis of its activity. In fish lysozyme levels do not remain constant but show variation with rearing environments, sex, bacterial species, and bacterial load (Minagawa et al., 2001; Yildiz, 2006). For instance, Japanese flounder showed higher lysozyme activity against *Pfiesteria piscicida* and *Vibrio anguillarum* compared *to Edwardsiella tarda* (Minagawa et al., 2001). Similarly, Šimková et al. (2020) reported higher lysozyme activity in roach during spring compared to autumn. For avoiding environmental and sex effects, we conducted an experiment in controlled conditions on the fingerlings of all cross-types of the same age and challenged all groups with the same pathogen, i.e., *A. hydrophilia*.

Our results of higher lysozyme activity in RR cross-type (*L. rohita*) before and after challenge to *A. hydrophilia* is in accord to literature (Nayak et al., 2011; Dash et al., 2014;

Kumar et al., 2018; Mohanty and Sahoo, 2010) that indicated the comparatively higher immune response or immunity of *L. rohita* compared to *C. catla*. We also observed the highest lysozyme activity in RC hybrid followed by CR hybrids both before and after exposure to the pathogen. It seems both hybrids have a strong defensive mechanism (lytic activity) against bacterial invasion compared to both parental species. This could be explained by the heterosis effect, as lysozyme concentration in both hybrids showed high parent heterosis before (RC, ≈ 39%; CR, ≈ 20% (Fig 20) and after (RC, ≈ 64%; CR, ≈ 28%) challenge to a pathogen. Like our results, Zhang (2019) also reported significantly higher lysozyme activity in hybrid catfish (*Pelteobagrus fulvidraco*♀× *P. vachelli*♂) compared to all-male *P. fulvidraco*. Conversely, Šimková et al.(2015) observed a week effect of hybridization on lysozymes activity in F1 hybrid of two phylogenetically related cyprinids, common carp and gibel carp, and reported an insignificant effect of season and sex. Whilst F1 reciprocal hybrids of two non-congeneric cyprinid common bream (*Abramis brama*) and common roach (*Rutilus rutilus*) showed intermediate lysozyme concentration, somewhat higher in spring with no sex effect (Šimková et al., 2015).

Phagocytic activity and phagocytic index are the important parameters of nonspecific cellular defense systems (Secombes and Fletcher,1992; Jensch-Junior et al., 2006). Phagocytosis is the most ancient tool of defense against foreign materials (Dzik, et al., 2010). In fish, macrophages, monocytes, and granulocytes are major phagocytic cells while in some species neutrophils also show phagocytic activity. These cells contain hydrolytic enzymes (acid and alkaline phosphatases, peroxidases, and lysosomal enzymes) for killing the infectious agents. Many investigators reported higher Phagocytic activity and phagocytic index in different fish species in response to *A. hydrophila* infection e.g., *C. gariepinus* (Al-Khalaifah et al., 2020), *C. carpio* (Anbazahan et al., 2014), *L. rohita* (Palanikani et al., 2019; Sharma et al., 2010), goldfish (Rahman et al., 2001; Harikrishnan et al., 2010).

Like lysozyme concentration, the phagocytic activity and phagocytic index of *L. rohita* before and after challenge to *A. hydrophila* were higher than *C. catla* while both hybrids showed higher activity compared to both parental species. Moreover, many-fold higher values of HPH and MPH indicated the higher vigor of RC hybrid compared to CR hybrid against pathogens. Like our results against *Aeromonas hydrophila*, crossbred hybrid (*O. aureus*♂ ×

O. niloticus♀) showed the highest percentage of phagocytic activity and phagocytic index compared to purebred genotypes (El-Hawarry and Saad, 2011). However, against a similar pathogen, phagocytic activity of hybrid was higher than blue tilapia but comparable to and Nile tilapia (Cai et al., 2004). Similarly, phagocytic activity of hybrid (Japan abalone × Japan abalone) and Japan abalone was statistically similar but higher than Taiwan abalone (You et al., 2019). Moreover, a hybrid between *C. macrocephalus* and *C. gariepinus* has no significant difference.

Respiratory burst Activity (RBA) represents the quick discharge of reactive oxygen species (ROS) including hydrogen peroxide and superoxide radicals from the professional phagocytes. These oxygen-free radicals act as potent bactericidal agents. Respiratory burst activity trend in all cross-types like other immunity parameters also indicated the comparatively higher resistance of RC hybrids to disease-causing organism compared to other cross-type. Many fold higher values of high and mid parent heterosis also indicated more improvement in the defense system of RC hybrid. Literature revealed variable results concerning RBA of hybrid to parental species. For instance hybrid of common bream and common roach showed an intermediate level of RBA (Simkova et al., 2020), while RBA in hybrid (Japan abalone x Japan abalone) and Japan abalone was statistically similar but higher than Taiwan abalone (You et al., 2019). However, Liang et al. (2014) did not find any difference in RBA among parental species (*H. discus hannai* and *H. gigantea*) and their reciprocal hybrids.

In the present study, we also determined the IgM level (specific immunity) of all cross-types. IgM plays a significant role, both in the innate and adaptive immunity of fish **(**Kataoke et al., 1980; Schatz, 2007; Flajnik, 2010). Literature reveals the capability of fish to produced specific IgM-type natural antibodies against different antigens, however, the intensity of the response depends on environmental conditions and species (Magnadottir, 1998). Moreover, the effect of sex on the IgM level of cyprinids is also well documented. For instance, males of common carp (Rohlenová et al., 2011) and *Rutilus rutilus* (Šimková et al., 2020) showed a higher level of IgM compared to females. Here we used juveniles of all cross-types, thus determined the IgM level irrespective of sex, and observed a significant increase in serum IgM level in all cross-types of fish after 24 hrs pathogen injection. Like our results, many workers

reported a similar rise in IgM level in different fish after invasion to *A. hydrophila*, e.g., *sparus aurata* (Becerril et al., 2011), Nile tilapia (*O. niloticus*) (Magid et al., 2019; El- Gawad et al., 2019; Moustafa et al., 2020), hybrid red tilapia (*O. mossambicus* × *O. niloticus)* (Monir et al., 2020), striped catfish (*Pangasianodon hypophthalmus*) (Sirimanapong et al., 2014).

Like all other immunity indices, IgM level also showed a clear interspecific difference, i.e., higher levels in RC hybrids followed by CR hybrids, while CC cross-type showed the lowest level before and after injection of a pathogen. Limited literature is available to compare our results of IgM level of hybrids to parental species. However, contrary to our observation, after exposure to *A. hydrophila*, Zhang et al. (2019) did not observe any significant differences in IgM levels of catfish hybrid (*Pelteobagrus fulvidraco*♀ × *P. vachelli* ♂) compared to all-male *P. fulvidraco*. However, hybrids of common carp and gibel carp showed a higher IgM level than gibel carp but equal to common carp (Šimková, 2014), while *Abramis brama* × *Rutilus rutilus* hybrid showed an intermediate level of IgM (Šimková et al., 2020).

The total serum protein level in all cross types also followed the same pattern as observed for immunity parameters, i.e., RC>CR>RR>CC and the same trend as reported by Peyghan et al. (2010), i.e., significantly high protein value in infected fish compared to a healthy one. Similarly, Atlantic salmon infected with *A. salmonicida* also showed a significant increase in serum γ-globulin fraction and total protein (Magnadottir et al., 1992; Moyner, 1993). In contrast to our findings, some works reported a decrease in serum or plasma total protein in infected fish. For instance, Evenberg et al. (1986), reported reduced in γ-globulin and total serum protein levels in carp infected experimentally with *A. salmonicida*. Similarly, Nile tilapia and *L. rohita* infected with *E. tarda* (Benli and Yildiz, 2004; Mohanty and Sahoo, 2010), common carp with spring viremia (Rehulka, 1996), and *Pseudomonas fluorescens* (Yildiz, 1998) infection also showed a significant decrease in the total protein levels. The variation in results may be due to species difference or infection level. A post-infection increase in total protein level observed here and reported by others may indicate the activation of groups of serum protein that regulate the immune function and protect from ROS cellular damage (Zhang et al., 2019), however, a decrease in serum protein could be due to strong challenge and tissue injury that cause a decline

in some the acute phase protein (Mohanty and Sahoo, 2010). The higher serum total protein in hybrids compared to both parental species is parallel to immunity indices and indicating their higher immune response against pathogens

In the current study, we also investigated the disease resistance of all cross-types in terms of survival (%) after challenged with *A. hydrophila***.** During 20 days of challenge assay, both RC and CR cross-types showed higher survival (%), i.e., improved disease resistance compared to both parental species may be due to favorable genotype. Based on challenge test results it seems that hybrids harbor fewer parasites or less susceptible to pathogens. Like rohu-catla hybrid, channel catfish and abalone hybrids in the challenge test also showed a higher survival rate (%) than parental species (Dunham and Smitherman 1987; Shuang Liang et al., 2018). Similarly, two reciprocal hybrids of *xiphophorus variatus and X. maculatus* also demonstrated significantly lower infection levels than either parental stock (Walter, 2001). A similar finding has also come from mollusks where hybrids were found more resistant to pathogenic bacteria *(V. harveyi, V. alginolyticus, and V. parahaemolyticus)* injection compared to parental species (Idrovo et al., 2016). There are only a few studies on the evaluation of the resistance gained by interspecific hybrids relative to the parental species and literature showed variable results like intermediate resistance of channel × blue catfish hybrids to enteric septicemia compared to the parental species (Wolters et al., 1996); negative heterosis, in resistance to *Edwardsiella ictaluri* in the channel × blue catfish hybrid (Bosworth et al., 2004); no significant difference in resistance to *Ichthyophthirius multifiliis* between channel catfish and channel × blue catfish hybrid (Xu and Klesius, 2013; Xu et al., 2011) but like our findings, higher resistance to *Flavobacterium columnar* in channel × blue catfish hybrid than both parental species (Arias et al., 2012).

The studies on immune-genetic aspects of fish are not only of 'purely scientific' interest but also have substantial practical applications that could facilitate the development of preventive strategies against pathogens (LaPatra et al., 2001; Emmenegger and Kurath, 2008). Transcriptional analyses have helped a lot in understanding changes in the levels of immune-related proteins, which are present in a modest amount in the blood or tissue, especially in the absence of an assay system for analyzing them. Though the expression studies of immune-related

genes in Indian carps have recently been initiated (Mishra et al., 2009; Sahoo et al., 2009; Mohanty and Sahoo, 2010), but understanding of immune responses to hybridization of these species at the transcription level is limited. Here we also studied the expression pattern of some immunity-related genes (TNF-α, lysozyme G, and lysozyme C) in the liver and muscle of all cross-types after injection of a pathogen (*A. hydrophila*) and observed significant up-regulation in all cross-types and variation in the mRNA level of these genes among cross-types. Among all cross-types, RC hybrid followed by CR and RR before and after challenge showed significantly higher expression of all immune-related genes, while fingerlings of CC cross-type showed comparatively lower expression. It seems hybrids combine some immunity traits from parents and showed more immunity than their parental species

Knowledge of immunity genes and their level of temporal expression against pathogens is essential to understand the basic mechanism of immunity. Therefore, in the present study, we also studied the temporal and spatial expression of the selected immunity genes (TNF-α, lysozyme G, and lysozyme C) after challenging all cross-types to pathogenic bacteria. Although Mohanty and Sahoo (2010) also selected these genes along with other genes for the evaluation of immune response against *E. tarda* pathogen but here is the first-time report on these genes in CR and RC hybrids in comparison with *L. rohita* and *C. catla*. Moreover, expression pattern of these genes individually in *L. rohita* has also reported (Mulder et al., 2007).

Like many reports (Fugh et al., 2013; Fu et al., 2013), we observed higher expression of lysozyme C in the liver than the muscle of all cross-types. In the liver, up-regulation of these genes was noticed at early stages of infection, i.e., 6 hr after Intraperitoneal administration of *A. hydrophilla*. Many workers reported similar early up-regulation of lysozyme genes in different fish species after similar intraperitoneal injection of a pathogen (Bilodeau et al. 2005; Mohanty and Sahoo, 2009 & 2010; Zhang et al., 2019) and suggested that via this administration, pathogen get somewhat quick access in the bloodstream and cause the induction of immune-related genes within 6 hrs. However, in contrast to our results, Fu et al. (2013) reported up-regulation of similar genes in the liver of Asian sea bass at 12hr post-infection (*Vibrio harveyi*).

In the present study, although the lysozyme C gene in the muscle of all cross-types showed up-regulation at 6 hrs, nevertheless mRNA level compared to observed in the liver was many-fold low. This is in accord with literature that showed low expression of this gene in the muscle (Fu et al., 2013). Moreover, lysozyme C in muscle started down-regulation at 12 hrs than the liver where it remained show higher expression. The post-infection expression of lysozyme G unlike lysozyme C showed an almost similar level of expression in both muscle and liver but temporal expression followed the same trend that in muscle showed peak level at 12 hr post-infection and then down-regulation compared to the liver where it showed higher expression at 6hr and remained up-regulated up to 12 hrs. Here we did not observe any significant difference in the temporal and spatial expression pattern of both lysozyme C and G genes among all cross-types. However, all pairwise statistical comparison indicated a significantly higher lysozyme C expression in RC hybrid while intermediate expression in CR hybrid in contrast to lysozyme G which showed higher expression in reciprocal hybrids both before and after pathogen challenge, thus indicating LPH in both hybrids and MPH and HPH in F1 RC hybrid. This relatively higher level of heterosis in the expression of both lysozyme C and lysozyme G in muscle and liver of RC hybrids may indicate the higher disease resistance compared to parental species. Although many investigators reported post-infection up-regulation of lysozyme genes in different fish species like Japanese flounder (Hikima et al., 2001), orange-spotted grouper (Yin et al., 2003), mandarin fish (Sun. et al., 2006), yellow croaker (Zheng et al., 2007) and *C. idella* (Ye, et al., 2009), yet no previous study compared the expression of these genes in hybrids to parental species.

TNF-α is an inflammatory cytokine produced by professional phagocytic cells (macrophages/monocytes) during acute inflammation and helps in the recruitment and activation of phagocytes (Roca et al., 2008). Like lysozyme C, TNF-α also showed higher expression in the liver compared to the muscle of all cross-types. In both liver and muscle of all cross-types, up-regulation of this gene was observed at early stages of infection, i.e., 6 hr after administration of pathogenic bacteria. Expression of this gene remained high up to 12 hrs and subsequently showed down-regulation. Like our result, a progressive increase in TNF-α expression early of bacterial infection, followed by a down-regulation was also reported previously (Rojo et al., 2007). However, Dash et al. (2017) reported an increase in TNF-α expression up to 48 hrs post-

infection and then a gradual decrease. Similarly, Booth et al. (2008) also reported significantly higher expression of TNF-α at 48hrs in response to *E. ictaluri* infection and then gradual down-regulation in channel catfish families. The activation of these cytokines immediately after infection and then drop in their levels gradually might reflect a decrease in the bacterial burden or neutralization of the inflammation. Many investigators reported similar up-regulation of the TNF-α gene in response to pathogens in different fish species, e.g. Japanese flounder (Hikima et al., 2001), zebrafish embryos, and adults (Pressley, 2005).

Like other immune-related genes, the expression of TNF-α also did not show any significant difference in the temporal and spatial expression pattern among all cross-types. After infection, in all cross-types (hybrids and parental species), it showed a similar trend of up-regulation and down-regulation at all sampling times. However, all pairwise statistical comparisons indicated a significantly higher expression of TNF-α in the fingerlings of both hybrids compared to parental species both before and after challenged to *A. hydrophila***.** This trend was found at all sampling times in both liver and muscle samples of all-cross-types. Though both hybrids (RC and CR) showed LPH, MPH, and HPH heterosis in the spatial peak expression of TNF-α but RC hybrids showed relatively higher levels of positive heterosis than CR hybrid. No previous work is available to compare our results.

Data are scarce on the direct immunity comparison of parent species and their reciprocal hybrids. Investigating the immune response of cyprinid species and their hybrids Andrea Simkova (2014) reported that hybrids showed an intermediate immunity compared to parent species. While Xu and Klesius (2013), observed the immune protection against *Ichthyophthirius multifilis***,** however unlike our results, did not observe significant variation in parasite infection, serum anti-Ich antibody, or survival of fish between immunized channel catfish and the hybrid of blue × channel catfish. However, Wolters et al. (1996), observed an intermediate post-challenge-response of antibody in blue catfish male × channel catfish female hybrids and revealed that the introgression of resistance genes is very important for resistance against diseases. In the same manner, the results of the humoral immune response strongly support the preceding results of the non-specific immune response parameters measured in the current study. During the present study, the defensive ability or the immune status was

more prominent in hybrid progeny than pure species, which might be due to inheritance and results in immune-competent hybrid fish (Alter et al., 2017).

Rohu and catla are popular food fish, especially in Asian countries. Although, both species are the most cultivable and popular in the polyculture system, however, the economic importance, consumer preference, market demand, and nutritive value of rohu are comparatively higher than catla (FAO, 2009 &2013; Ahmed et al., 2020). Thus, here we also evaluated the impact of hybridization on nutritive value, i.e., chemical (crude protein, crude fats, ash, and moisture contents), amino acids, and fatty acids composition of the muscle of all cross-types. Literature revealed considerable variation in proximate composition among various species and even among different individuals belonging to the same species (Memon et al., 2010; Taşbozan et al., 2016; Hussain et al., 2018) and showed the strong effect of environmental and biological factors. Some workers reported the impact of environment like culturable conditions, farming system and fishing on the size of muscle fiber and contents of lipids and protein (Solari, 2006; González et al., 2009), while others observed the effect of biological factors or intrinsic factors like species, strain, age, body weight, feed, sex and migratory behavior (Suárez et al., 2002; González et al., 2009). Among environmental factors diets or source of food showed more impact on the nutritional composition compared to other factors (Herawati et al., 2018; Desta et al., 2019). To the best of my knowledge, except Olaniyi et al. (2016), no literature is available that showed comparative muscle proximate composition of hybrids with their parental species.

In the present study, for the proximate composition of muscle, we selected the juvenile fish of similar age after rearing in a communal pond and found significantly ($p=0.001$) higher crude protein content in *L. rohita* followed by RC hybrid, CR hybrid, and *C. catla*, respectively. It is in accord with literature that reported the highest percentage of protein in rohu in comparison to other carps (Mukundan et al., 1986). Although, reciprocal hybrids of *C.* and *H. bidorsalis* also showed somewhat intermediate protein contents but showed higher variation within hybrids compared to parental species (Olaniyi et al., 2016). However, in the present study, no such variations were observed and both hybrids and parental species showed a similar level of variation in muscle protein contents. The intermediate contents of protein observed here and reported by Olaniyi et al. (2016) may indicate the hybridization effect.

Moreover, the maternal effect was also observed in the present study, as RC hybrids showed higher content than CR hybrids.

Fat content of all cross-types found < 5%, indicating lean fish (Osman et al., 2001). Such a class of fish stores 50-80% of its fat in the hepatopancreas as triacylglycerol (Jacquot, 1961). According to Osman et al. (2001), the flesh of lean fish is white due to a high level of moisture. Similar, higher moisture and less than 5% fat have also been observed in many fish species (Zmijewski et al., 2006). Fat content observed in the current study significantly differed ($P < 0.05$) among cross-types, and hybrids showed the same intermediate pattern between *C. catla* and *L. rohita*. The highest fat content observed in *L. rohita* followed by RC hybrid, CR hybrid, and *C. catla*, respectively. However, in contrast to our result, reciprocal hybrids of catfish (*C. gariepinus* × *H. bidorsalis*) showed higher levels of fat contents than parental species, which may be due to species differences. Literature revealed a significant difference in lipid contents among species and even among strains of similar species (Erickson, 1992), and indicating a strong effect on both flavor and taste of fish meat (Heinz and Hautzinger, 2007). Here, the intermediate value indicated heterosis and improvement in the quality through hybridization.

The ash and moisture content also varied significantly ($P < 0.05$) among cross-types. Moisture content was high in the muscle of catla**,** low in rohu, and intermediate in hybrids. Our finding also showed the impact of hybridization on the amount of inorganic components of the fish body. We observed maximum ash content in *C. catla* followed by CR hybrid, then RC hybrid, and least in *L. rohita*. Unlike our results, Olaniyi et al. (2017) found high moisture content in catfish hybrids with high individual variation compared to parental species (*C. gariepinus* and *H. bidorsalis*. The water content in the meat has a profound effect on the processing quality and the uniformity of the final product (Heinz and Hautzinger, 2007). Here both hybrids (CR and RC) had an intermediate level of moisture contents between parental species, and low level of individual variation compared to catfish hybrid. Again showed some improvement in flesh quality as compared to *C. catla*.

A characteristic fish flavor is also related to a free amino acid level found in the muscle tissue. Additionally, some amino acids like glutamate, histidine, and glycine substantially contribute to the taste, texture, and quality of fish meat (Li et al., 2009). It is well established that the nutritional value of meat is characterized not only by protein content but also by the composition of amino acids. In the present study, muscle amino acid composition showed a higher level of essential amino acids in *C. catla* followed by RC hybrid, then *L. rohita*, and least in CR-hybrid. Like our results, Mohanty et al. (1991) also reported higher levels of essential amino acids in *C. catla* compared to *L. rohita*. However, no previous studies address the impact of hybridization on the amino acid profile of fish. Here we did not get any clear picture because CR hybrid showed the least contents of NEAA and highest level of EAA compared to RC hybrids where NEAA content was less than catla but higher than other cross-types, while EAA level was higher than catla but lower than other cross-types. Further molecular level study is required to reach any conclusion.

Fatty acid contents in different fish species vary in chain length from C14–C24 and degree of saturation to un-saturation and ω-6 to ω-3 ratios. The variation in the fatty acid profile of fish within and between species could be due to the difference in their natural and prepared diets especially in the quantity and quality of the dietary source of lipid (Khan et al., 2015; Afridi et al., 2018) along with many others extrinsic and intrinsic factors (Mráz and Pickova, 2011; Qiu et al., 2017; Afridi et al., 2018; Zhang et al., 2019). To avoid these factors, juvenile fish of all cross-types of the same age group, reared in the communal earthen pond on the same prepared diet were selected for the analysis of the fatty acid profile and found variations in the muscle contents of SFAs, USFAs, PUFAs and ω3to ω6 ratio among parent species and hybrids.

The observed muscle fatty acid composition of *L. rohita* was in accord with the literature. Like our observations, Afridi et al. (2018) also reported similar lower concentration of SFA (saturated fatty acids), higher contents of MUFA (monounsaturated fatty acids), and PUFA (polyunsaturated acids) especially docosahexaenoic acids (DHA) and eicosapentaenoic acid (EPA) and ω3/ω6 ratio in similar size of *L. rohita*. Fatty acid composition of fish muscle indicates the nutritional quality and oxidative stability of lipids. For instance, consumption of SFAs is associated with higher LDL-cholesterol level and coronary heart disease (Santos et al.,

2013; Fernandes et al., 2014; Rodrigues et al., 2017) while monounsaturated (MUFA) improve the lipids profile (Liu et al., 2013). Similarly, polyunsaturated fatty acids (PUFA) have anti-arrhythmic, anti-inflammatory, antithrombotic, hypolipidemic, and vasodilatory properties (Simopoulos, 2016; Sokoła-Wysoczańska et al., 2018). Here, higher contents of MUFA and PUFA in the muscle of rohu compared to catla which showed higher levels of SFA support the previous reports (FAO, 2009 & 2013) and indicating higher nutritional quality of rohu compared to all other cross-types.

Fatty acid profile of reciprocal hybrids showed clear intermediate levels of SFA, MUFA, and PUFA and a lower level of ω3/ω6 ratio compared to both parental species. No literature is available to compare the results of reciprocal hybrids and the impact of hybridization on the fatty acid profile of fish. In the present study, all cross-types were on the same prepared feed and of similar age, thus the observed variation in fatty acid profile may be related to their metabolism and genotype of each cross-type (Tocher, 2003).

Generally, the balance of ω6 and ω3 fatty acids is important because they are metabolically and functionally distinct and are not inter-convertible, and often have important opposing physiological effects, (Simopoulos, 2016). The ω-6 fatty acids increase the membrane permeability which then increases intracellular triglycerides, while omega-3 fatty acids suppress lipogenic enzymes and increase beta-oxidation which reduces the fat deposition (Simopoulos, 2016). Omega-6 and omega-3 fatty acids work together to create balance, homeostasis, within the body. According to some scientists, a 1:1 ratio of ω -3 to ω -6 fatty acids is good from a nutritional point (Simopoulos, 2016) while others suggested 1:1 to 1:5 ratios with a predominant amount of EPA and DHA, best for a healthy human diet (Osman et al., 2001). Here hybridization showed a somewhat negative effect on the ω -3 to ω -6 ratio, i.e., both hybrids showed < 1 ratio compared to parent species that showed >1ratio. Moreover, RC hybrids showed a comparatively higher ratio compared to CR hybrids. Overall results of proximate composition, amino acid profile, and fatty acid profile indicated the higher nutritional value of RR-cross-type (rohu) followed by hybrids. Thus indicating the effect of interspecific hybridization on the nutritive value of fish.

Conclusion

In conclusion, this study demonstrated the intermediate growth performance, thermal tolerance, nutritive value, and morphological features while improved immunity and disease resistance of both F1 hybrids (rohu-catla and catla-rohu) compared to both parental species (*L. rohita* and *C. catla* were observed. These results also indicated the higher disease resistance, immunity, thermal tolerance, and nutritive value of the F1 RC hybrid compared to F1 CR hybrids which showed a higher growth rate. Moreover, each hybrid showed an identical phylogenetic relationship with the mother species, i.e., CR hybrids showed the minimum distance with CC, and RC hybrid showed the minimum distance with RR. These results suggest that both hybrids have the potential to be introduced in aquaculture for improving production per unit area.

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Effect of acclimated temperature on thermal tolerance, immune response and expression of HSP genes in *Labeo rohita, Catla catla* and their intergeneric hybrids

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ABSTRACT

The ability of a species and population to respond to a decrease or an increase in temperature depends on their adaptive potential. Here, the critical thermal tolerance (CTmax and CTmin) of four populations: *Labeo rohita, Catla catla,* and their reciprocal hybrids *L. rohita*♀× *C. catla*♂ (RC) and *C. catla*♀ × *L. rohita*♂ (CR) being acclimatized at four acclimation temperatures (22, 26, 30 and 34 °C) were determined. All populations indicated substantial variations ($P < 0.05$) in CTmax and CTmin values. *L. rohita* displayed, comparatively the highest CTmax with largest total and intrinsic polygon zones as well as the upper and lower acquired thermal tolerance zones followed by RC and CR hybrids, while *C. catla* showed significantly the highest CTmin value and the smallest intrinsic and acquired thermal tolerance zones. Both hybrids illustrated low parent heterosis (≤11%). Additionally, the highest expression of Hsp70 and Hsp90 (heat shock proteins) genes, serum lysozyme level, respiratory burst activity and lowest lipid peroxidation level under lower and higher temperature shock further illustrated strong physiological mechanism of *L. rohita* in contrast to *C. catla,* to deal with acute temperature, while hybrids, especially F1 RC hybrid appeared as a good option to replace *C. catla* in relatively higher and lower temperature areas.

1. Introduction

Global climatic changes, including temperature variations are now been considered as the major threat to biodiversity and can possibly affect the aquaculture sector negatively, by lowering fish productivity worldwide (Ficke et al., 2007). Climate change may lead to creation of physical or physiological barriers (Walther et al., 2002; Pearson and Dawson, 2003) that might limit the habitat's shift of independently mobile organisms and in turn leads to loss of genetic diversity (Ezard and Travis, 2006). The environmental temperature is the key factor that alters the various aspects of fish biology; distribution, behavior, fitness, and performances (Pörtner and Farrell, 2008). Although thermal preferences are species-specific and genetically inherent (Anttila et al., 2013), but other endogenous and abiotic factors like age, sex, weight, food availability, density, pathogens, season, water quality and light intensity also have an intense impact (Díaz et al., 2002).

The impact of seasonal and climate changes on thermal tolerance as well as life of the organism has widely been recognized (Bozinovic et al., 2011; Yanik and Aslan, 2018). The increase or decrease in temperature beyond optimum level cause physiological changes/disturbance which ultimately leads to the death of an organism. To cope with these changes, fish have limited options i.e., to move to a more appropriate habitat or adapt new environmental conditions, through the expression of phenotypic plasticity (Schulte et al., 2011; Fox et al., 2019). Most of the species have ability to modify their thermal tolerance limit and adjust to the new temperature regimes by modifying physiological functioning through acclimation (Angilletta et al., 2009; Seebacher et al., 2015). Nevertheless, physiological plasticity in thermal tolerance varies among species or even among strains, thus effecting their adaptation (Stitt et al., 2013; Fu et al., 2018).

Temperature rise is beneficial for aquaculture production to a certain range, as it enhance metabolism and growth rate, along with reduction in the maturation or culture period (Boltaña et al., 2017; Islam et al., 2019), however, increase in temperature beyond the optimal limit can

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surge metabolic stress, increase the oxygen demand (Claësson et al., 2016) and negatively impact the immunity (Dominguez et al., 2004; Abram et al., 2017). It is well documented that the expected 1–4 °C rise in temperature due to global warming over the following century will also affect aquatic ecosystems (Ficke et al., 2007; Pachauri et al., 2014). Like higher temperature, suboptimal temperature also impaired the health of fish by effecting metabolism, slowing or shutdown immune response mechanism and increasing susceptibility to pathogens (Liang et al., 2014; Abram et al., 2017).

Many studies demonstrated the variation in thermal tolerance, both within species/strains (McDermid et al., 2012; Stitt et al., 2013; Syed et al., 2018) and among species (Chatterjee et al., 2004; Das et al., 2004) and proposed intraspecific and interspecific hybridization. Hybridization is an effective method in aquaculture to improve the growth, disease resistance, survival etc. of fish and shellfish (Wang et al., 2019). Many investigators also used this method to improve the thermal tolerance and observed variable results. For instances F1 hybrids of *Morone chrysops*♀ and *M. saxatilis*♂, *Haliotis discus hannai*♀ and *H. discus discus*♂, *H. discus hannai*♀ and *Halitotis kamtschatkana*♂, *H. discus hannai*♀ and *H. giganteanꝏ, Ctenopharyngodon idella*♀ and *Hypophthalmichthys nobilis*♂ showed stronger thermal tolerance than both parents (Bettoli et al., 1985; Bartley et al., 2000; Liang et al., 2014) while observed thermal tolerance of F1 hybrids of *Esox lucius* and *Esox masquinongy*, *Salvelinus fontinalis and S. namaycush* (Scott, 1964; Goddard and Tait, 1976) was intermediate between parents. These examples have confirmed the hybrid heterosis in thermal tolerance. However, several studies have demonstrated no heterosis among interspecific and intraspecific hybrids like low temperature tolerance of *Sparus sarba* × *Acanthopagrus schlegeli* F1 hybrids was more or less identical to the parent (Kitajima and Tsukashima, 1983). Similarly, pure and F1 hybrid populations of brook trout *Salvelinus fontinalis* did not show any pronounced difference in CTmax values.

Generally, thermal tolerance of aquatic ectotherms has been calculated through the critical thermal limits. At this point, fish may show a combination of physiological and biochemical features (Somero, 2010) including onset of muscle spasms and loss in the righting response. In the laboratory, the upper and lower temperature tolerance limit of fish could be quantified by adopting a critical thermal methodology (CTM) (Cowles and Bogert, 1944), that involve exposure of fish to a continuous and constant decrease or increase in temperature until a sub-lethal or near-lethal endpoint, i.e., disorganization of locomotor activity of fish is attained (Eme and Bennett, 2009). Survival beyond the critical thermal maximum (CTmax) is limited by time, due to the insufficient ATP yield from anaerobic metabolism. Temperature intervals between the critical thermal limits and optimal temperature are called Pejus, a transitory temperature where protection mechanisms to cope with the generation of reactive oxygen species (ROS) are activated (Rodríguez-Fuentes et al., 2017).

Nowadays, genetic characterization of thermal resistance is getting importance in fish culture. Unfortunately, little is known about the genetic basis and mode of inheritance of this phenomenon in most of fish species. Nevertheless, many investigators suggest it a heritable trait that might be controlled by single or polygene (Doyle et al., 2011; Anttila et al., 2013), probably passed on by sex-linked inheritance (Nakajima et al., 2009). The present novel study is the first ever laboratory trial to elucidate the thermal tolerance and physiological responses of fingerlings of *L. rohita, C. catla,* and their intergeneric hybrids acclimated at different temperatures. According to some investigators, genetic variability via hybridization could improve population responses to change in temperature, under the concept of natural selection (Stockwell et al., 2003; Pickup et al., 2013). Therefore, this study was designed to evaluate phenotypic plasticity and adaptive potential of the parental fish and their reciprocal hybrids to various temperature regimes so as to improve and disseminate their lucrative position in aquaculture practices.

2. Materials and methods

2.1. Fish sampling and acclimatization

Fingerling of *L. rohita, C. catla,* CR hybrid (right pectoral fin clipped), and RC hybrid (left pectoral fin clipped) of similar age group, average body weight 24.45 ± 2.0 g and length 12.3 ± 0.5 cm) were harvested from earthen pond and stocked in the circular water tanks having venturi drain, for 5 days prior shifting to experimental aquaria for initiating experiments. All populations were fed with 35% protein commercial diet, twice a day at *ad libitum.*

2.2. Experimental design

2.2.1. Thermal tolerance

A 4x4 factorial design, consisting of four populations (*C. catla, L. rohita*, CR hybrid and RC hybrid) and four acclimated temperatures was conducted to determine the thermal tolerance of each population. The acclimatized fingerlings of each population were shifted from tank to experimental aquaria (120 × 60 × 60 cm^3, 432L), having aerators to maintain DO level (5.5 mg/L), immersible water heaters (microcomputer temperature controller Jiayu MI-300 W) and a 1/4 HP local made Tank Chiller to manage water temperature at desirable degrees. In order to avoid effect of extraneous variables, 5 fingerlings of each population were stocked in the same aquarium (20 fingerlings/aquarium). The thermal tolerance trial was conducted in replicates of three and 6 aquaria (3 for CTmax and 3 for CTmin) of every population (15 fingerlings/population/CTL) were used for each acclimated temperature. The water temperature of each aquarium was decreased or increased gradually at the rate of 1 °C/day to reach the test acclimation temperatures (22, 26, 30, 34 °C) by following previously reported procedure (Syed et al., 2018). Later the fish were hold for a period of 30 days at a specific acclimated temperature before starting the trial. The acclimation temperatures for the trial were selected by following literature on *C. carpio* and *L. rohita* (Chatterjee et al., 2004; Das et al., 2004; Syed et al., 2018). Throughout the experiment, the concentration of dissolved oxygen (DO) and temperature were monitored. Moreover, the experiment was conducted in the semi-static conditions and 20–30% water was exchanged with freshwater, depending on the turbidity and water quality of the aquaria. Afterwards, fish were starved for a day before the initiation of the thermal tolerance assay.

2.2.2. Thermal tolerance evaluation

For population-based thermal tolerance, Critical thermal methodology (CTM) reported by other investigators (Das et al., 2004; Syed et al., 2018) was adopted. Briefly CTmax and CTmin tests were conducted by gradual increment and reduction in the water temperature by 0.3 °C/min of each aquarium set at acclimated temperature until fish showed loss of equilibrium i.e., unable to maintain dorso-ventral orientation for about 1 min (Eme and Bennett, 2009). Lowest and highest temperature points at which LOE of fish was observed were considered as critical thermal minima (CTmin), and critical thermal maxima (CTmax) of particular population, respectively. Aeration was well maintained throughout the experiment, while instantaneous temperature fluctuations were noted with digital thermometer attached to each aquarium. The upper and lower thermal limit of each population was calculated by noting thermal tolerance of every individual in replicate group. Subsequently, grand, mean, of collective replicate endpoints was considered as the CTmax and CTmin of the population.

2.2.3. Thermal tolerance polygon

Method reported in detail by Eme and Bennett (2009) was adopted for the construction of ecological thermal polygons and calculation of intrinsic and acquired thermal zones of *L. rohita, C. catla* and their hybrids.

2.2.4. Physiological response

For comparative evaluation of stress and immune response of *L. rohita, C.catla,* and their reciprocal hybrids (RC and CR hybrids) after higher and lower temperature shock, second set of experiment in replicate of three under similar setup as mentioned above was conducted. Here 45 fingerlings of each population were evenly distributed in 9 aquaria (20 fingerlings/aquarium with 5 fingerlings/population) and acclimated at 27 °C± 0.5 for 30 days. Afterwards fish of three aquaria were exposed to 12 °C while other fish were exposed to 37 °C. The DO level was maintained and temperature was increased or decrease as described previously. Fish were captured after 6 h of attaining assigned temperature and samples were collected for evaluating the response of each population under thermal stress.

2.2.5. Tissue and blood collection

For collection of serum and hepatopancreatic tissue, 45 fingerlings of each population (15 each from 27 °C, 12 °C and 37 °C temperature exposed groups) were anesthetized with buffered MS222 (70–80 mg/L), and blood was collected from the caudal vein by using 2 ml heparinized syringe (24G). Blood was allowed to clot at ambient temperature. Subsequently, centrifuged for 10 min at 2000×*g* and separated serum was saved in microtube at 4 °C. Fish were dissected and their hepatopancreatic tissues were collected aseptically. For determination of heat shock protein genes expression, about 50 mg tissues of randomly collected 9 fingerlings/high, low and acclimated temperature (3 fingerlings/aquarium) were immediately preserved in RNA later at −80 °C, while remaining tissues of 3 fingerlings/aquarium/population were pooled and hepatopancreas of other 6 individuals/population/temperature were collected separately and stored at −20 °C for lipid peroxidation assay.

2.3. Biochemical analysis

2.3.1. Respiratory burst activity

Anderson and Siwicki (1995) method was adopted for determination of respiratory burst (rapid release reactive oxygen species) by using fresh heparinized blood of randomly collected 3 fish/-aquarium/population). The level of reduced nitroblue tetrazolium was noted at 540 nm.

2.3.2. Lysozyme activity

Serum lysozyme activity (9 samples/population/temperature) was determined by adopting standard method reported earlier (Ullah et al., 2018).

2.3.3. Lipid peroxidation

Wright et al. (1981) method described in detail earlier (Ullah et al., 2014) was adopted for the determination of lipidperoxidation level in the hepatopancreas.

2.4. Extraction of total RNA and cDNA synthesis

The detailed procedure reported earlier (Amir et al., 2018) was adopted for extraction of total RNA from samples and conversion into cDNA for qPCR.

2.4.1. Designing of primers

The primers used for the study were self-designed by using Oligo Primer Analysis Software version 1.1.2 based on the mRNA sequence of the respective genes of *L.rohita* obtained from gene bank NCBI (www.ncbi.nlm.nih.gov). The designed primers were manufactured from Humanizing Genomics Macrogen. The specificity of each primer was checked by running a simple PCR reaction and performing gel electrophoresis, while the quality of cDNA and its compatibility with primers were checked by performing PCR of each sample in duplicate in at least two independent runs. List of primers used for evaluating the expression of Hsp genes are given in Table 2.

2.4.2. RT – qPCR for gene expression

For evaluation of the relative expression of heat shock proteins Hsp70 and Hsp90 genes in the hepatopancreas, quantitative real time PCR was conducted. Initially, PCR conditions and cycle number were optimized for Hsp genes. Each PCR reaction was run in standard of 20 µl reaction mixture contains 1.6 µl of diluted cDNA, 0.4 µl of forward and reverse primer, 10 µl of SYBER green and 7.6 µl of H_2O. RT - qPCR reaction condition included an initial denaturation at 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s and subsequently, 62 °C for 15 s. The 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* 18s Housekeeping gene, primer shown in Table 2. The relative variation in gene expression was calculated by the standard ΔΔCT method (Pfaffl, 2001).

2.5. Statistical evaluation

R-software was used for dawning thermal polygon and determination of intrinsic and acquired thermal zone of each population. Whilst computerized statistical software package for social sciences (Version 20, Inc. Chicago, USA) was used for conducting two ways analysis of variance followed by LSD post hoc test for comparing the results of Critical thermal limits and biochemical indices among populations, parent species (*L. rohita, C. catla*) and their intergeneric hybrids (RC and CR) at different temperatures. The relationship between the acclimatization temperature and thermal tolerance of each population was determined by simple linear regression. All statistical analysis was based on 5% level of significance.

3. Results

Statistical analysis showed significant differences in CTmax values among populations, *L. rohita*, *C. catla*, CR and RC hybrid ($n = 15$, ANOVA, $F_{3,\ 240} = 403.68$, $p = 0.001$) at a different acclimated temperature ($F_{3,\ 240} = 5.28$, $p = 0.001$), while pop × acclimated temperature significant interaction ($F_{3,\ 240} = 3.50$, $p = 0.001$) indicated the manner in which the populations responded to the acclimated temperature varied. Similar results were observed for CTmin values, i.e., all populations showed significant difference ($n = 15$, ANOVA, $F_{3,\ 240} = 564.47$, $p = 0.001$) at a different acclimated temperature ($F_{3,240} = 5.62$, $p = 0.001$) while population × acclimated temperature significant interaction ($F_{3,240} = 25.39$, $p = 0.001$) indicated how the effect of both variables depend on each other. The close observation of data indicated that values of CTmax and CTmin increased significantly, with increasing acclimation temperatures ($P < 0.05$), therefore, at higher acclimation temperatures, the fingerling of *L. rohita, C. catla*, CR and RC hybrid had higher CTmax and CTmin values (Table 1). Subsequent to CTM trials, fish did not show any mortality during a 24 h observation period.

Moreover, regression analysis revealed a comparatively less gain in the tolerance of *C. catla* with changes in acclimation temperature as compared to *L. rohita* and hybrids (CR and RC). For every 1 °C vary in acclimation temperature, both *L. rohita* and RC hybrid gained 0.66 and 0.64 °C of the upper tolerance limit while 0.49 and 0.48 °C, respectively of the low tolerance limit. Unlike *C. catla,* both hybrids displayed more upper and lower tolerance with change in acclimation temperature. Yet, in contrast to *L. rohita*, RC hybrid showed comparable lower and less upper tolerance to change in acclimation temperature while acclamatory response of CR hybrid with respect to *L. rohita* was less in upper temperature tolerance, but greater in lower temperature tolerance. Furthermore, highly significant regression of CTmin and CTmax on acclimation temperatures for all populations ($p < 0.001$) indicating 0.94–0.99% thermal tolerance variability (Fig. 1a and b).

Table 1
Critical thermal maxima (CTmax) and critical thermal minima (CTmin) of *L. rohita, C catla* and their intergeneric hybrids (CR and RC) acclimated to four different temperatures.

	Acclimation temperature (22 °C)				Acclimation temperature (26 °C)			
	Parent species		Hybrids		Parent species		Hybrids	
	L. rohita	*C. catla*	CR	RC	*L. rohita*	*C. catla*	CR	RC
CTmax(°C)	37.42 ± 0.04[a]	35.62 ± 0.12[d]	35.92 ± 0.07[c]	36.62 ± 0.07[b]	40.09 ± 0.12[a]	38.04 ± 0.04[d]	38.65 ± 0.03[c]	39.2 ± 0.04[b]
CTmin(°C)	10.40 ± 0.14[a]	10.29 ± 0.11[b]	8.94 ± 0.11[c]	8.21 ± 0.06[d]	10.53 ± 0.02[c]	11.45 ± 0.33[a]	11.01 ± 003[b]	9.98 ± 0.33[d]
	Acclimation temperature (30°C)				**Acclimation temperature (34°C)**			
CTmax(°C)	42.56 ± 0.13[a]	40.61 ± 0.30[d]	41.27 ± 0.02[c]	41.96 ± 0.02[b]	45.40 ± 0.16[a]	42.90 ± 0.12[d]	43.33 ± 0.14[c]	44.29 ± 0.08[b]
CTmin(°C)	11.98 ± 0.03[d]	14.38 ± 0.03[a]	13.15 ± 0.04[b]	12.55 ± 0.06[c]	14.45 ± 0.11[c]	15.39 ± 0.07[a]	14.90 ± 0.06[b]	13.76 ± 0.08[d]

Data presented as Mean ± SE. One-way ANOVA followed by an LSD post hoc test shows a comparison between populations. All means with a different lowercase superscript in the same row indicate significance difference ($P \leq 0.05$). CR = *C.catla*♀×*L. rohita*♂ hybrid; RC = *C. catla*♂ ×*L. rohita*♀ hybrid.

3.1. Thermal tolerance zone

Among all populations, *L. rohita* exhibited the largest polygonal area followed by RC hybrid (Fig. 1a and b). All populations polygon area was ranging from 316.98 °C^2 (*C. catla*) to 360.41 °C^2 (*L. rohita*). The displayed polygon area of RC (352.85 °C^2) and CR (333.47 °C^2) hybrids was at least 36.44 (11.52%) and 17.06 (5.39%) greater than *C. catla* respectively. The results reflect low parent heterosis as *L. rohita* gained the greater amount of upper and lower tolerance through acclimation while *C. catla* acquired the least upper and lower thermal tolerance limits.

3.2. Biochemical indices

Before thermal shock or at 27 °C, all populations showed significant difference ($P < 0.05$) in serum lysozyme activity, respiratory burst activity and liver lipid peroxidation level (Table 3). RC hybrids followed by CR hybrids showed significantly higher levels of lysozyme and respiratory burst activity while *C. catla* showed the lowest values. The lipid peroxidation levels in RC hybrids, *L. rohita* and CR hybrids were statistically similar ($P > 0.05$) and lower than found in *C. catla*. Exposure of fingerlings to higher (38 °C) and lower (12 °C) temperature caused a significant increase in the LPO levels, lysozyme and respiratory burst activity. All populations followed similar rank in their responses as observed at 27 °C.

The expressions of Hsp70 and Hsp90 genes in the hepatopancreas of all populations were significantly ($p < 0.05$) up-regulated after thermal stress (low and high temperature shock). One way ANOVA followed by LSD post hoc test revealed that expressions of both Hsp were significantly different among populations (Fig. 2, Hsp70, n = 9, $F_{3,108}$ = 13.067, p = 0.001: Fig. 3, Hsp90, n = 9, $F_{3,108}$ = 9.47 p = 0.001) at different temperatures (Hsp70, n = 9 $F_{2,108}$ = 63.36, p = 0.001 and Hsp90, $F_{2,108}$ = 68.30, p = 0.001) Moreover, significant interaction between populations and temperature (Hsp70, $F_{6,108}$ = 5.33, p = 0.001 and Hsp90, $F_{6,108}$ = 2.30, p = 0.01) indicated the manner in which the all populations responded to the change in temperature. The close observation of the data indicated statistically comparable and many folds higher expression of genes related to Hsp70 and Hsp90 in *L. rohita* and RC hybrids as compared to *C. catla* after thermal shock.

4. Discussion

The most important attributes for the sustainable development of modern fish culture systems are fast growth, disease resistance and higher thermal tolerance of stocks and through interspecific hybridization superior genetic and phenotypic variations of these traits can be generated. Here, trials have been conducted to explore these variations for thermal tolerance and the strength to cope with the change in temperature (thermal stress) in *L. rohita*, *C. catla* and their reciprocal hybrids (RC and CR hybrids). Our study revealed significant variations among them in thermal tolerance at four acclimation temperatures; 22, 26, 30 and 34 °C and in physiological responses at higher and lower temperature assault.

Previously, many studies showed that thermal tolerance of fish is mostly dependent on the acclimation temperature (Das et al., 2004; Debnath et al., 2006; Eme and Bennett, 2009; Syed et al., 2018), while others reported the influence of many other factors like species, size, age, condition factor and pollution on this trait (Beitinger et al., 2000; Das et al., 2004; Nakajima et al., 2009). In the present investigation, the experimental setup and acclimation temperatures were set by following the studies on *L.rohita* (Das et al., 2004; Syed et al., 2018), while influence of different external factors was avoided by conducting experiment in replicate of three in communal aquaria on the same age fingerlings of both species, *L. rohita*, *C. catla* and their hybrids (RC and CR). Furthermore, three months before conducting thermal tolerance assays, fingerlings of all groups were reared in communal earthen ponds. Despite providing similar environmental conditions, before and during assay, the parent species and their reciprocal hybrids showed significant difference in CTmax and CTmin values, may be due to genetic variation relating to expression of this trait (Stockwell et al., 2003; Nakajima et al., 2009; McDermid et al., 2012).

Here, CTmax and CTmin values of *L. rohita, C.catla* and both hybrids increased proportionally to rise in acclimation temperatures (Table .1). Like our results, many other investigators also reported positive relation between acclimation temperature and thermal tolerance in different fish species, e.g., *C. carpio,* yellowtail catfish, (Chatterjee et al., 2004), advance fingerlings of Indian major Carps (Das et al., 2004, 2005) and *P. pangasius* (Debnath et al., 2006). Furthermore, higher CTmax and lower CTmin values of *L. rohita* at all acclimation temperature as compared to *C. catla* in the present study is also in accord to previous reports (Chatterjee et al., 2004; Das et al., 2004), indicating species specific difference in thermal tolerance. Though, some differences in CTmax and CTmin values of both species from the previous investigations have been observed, but it may be due to the differences in acclimation temperature, age and size of fish (Das et al., 2004; Nakajima et al., 2009).

Our results also indicated thermal tolerance differences within hybrids (RC and CR) and between hybrids and parent species. Both hybrids displayed more upper and lower temperature tolerance in contrast to *C. catla* with change in acclimation temperature, while acclamatory response of CR hybrid with respect to *L. rohita* was less in upper temperature tolerance, but greater in lower temperature tolerance. Nevertheless, no literature is available on the thermal tolerance of CR and RC hybrids to compare our findings, but our results support various reports illustrating thermal tolerance difference between fish species as well as even among different strains (McDermid et al., 2012).

Thermal tolerance polygons give a very good idea about the thermal physiology, ecology and distribution strategy and thermal niche along with the optimal culture conditions of the species (Das et al., 2004; Dülger et al., 2012). Here, of the four groups (parent species and their

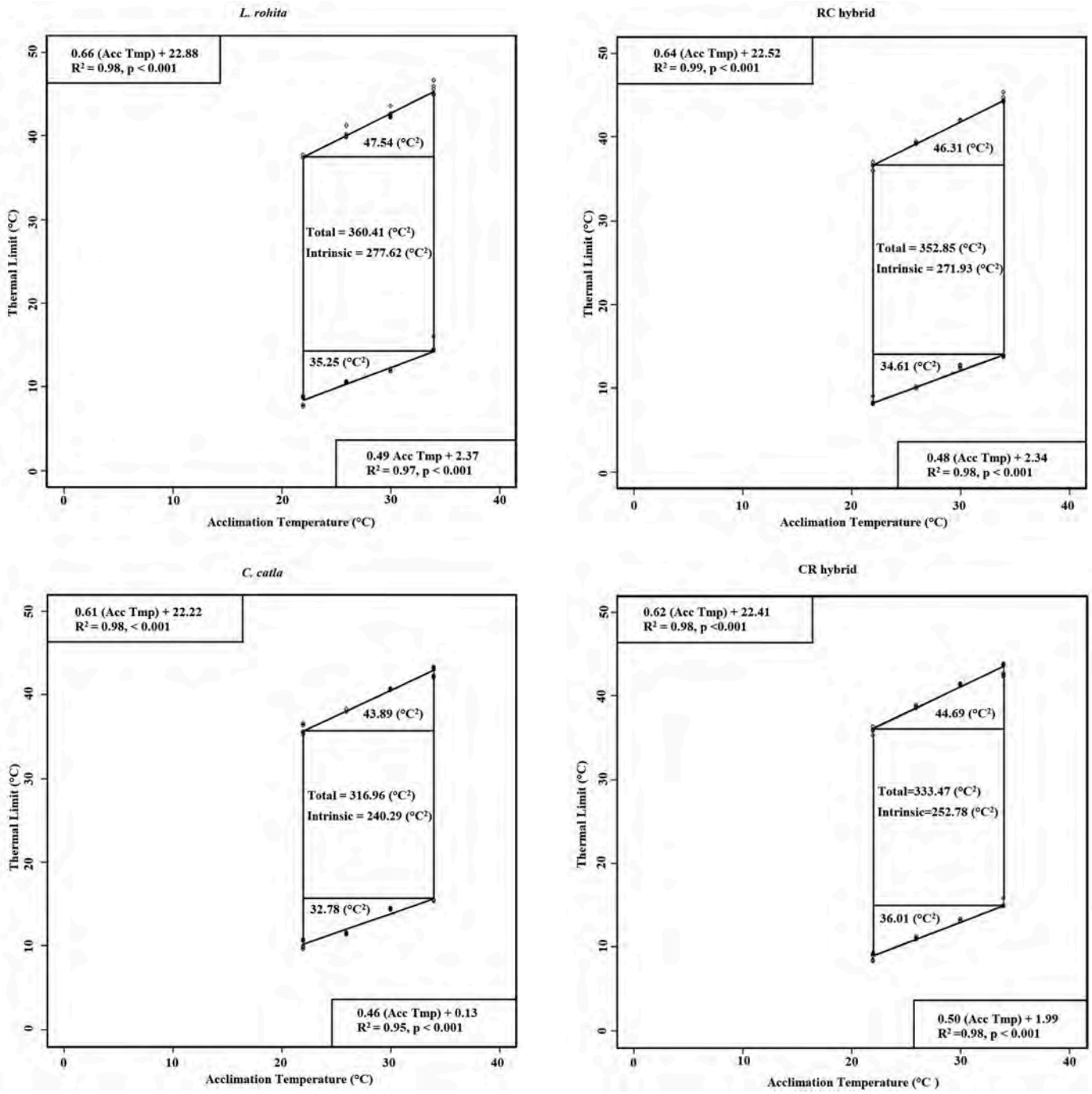

Fig. 1a. Thermal tolerance polygon created by using CTmax and CTmin data. It shows the thermal tolerance zone of *L. rohita*, and *C. catla*, acclimated to four different temperatures (22, 26, 30 and 34 °C). Polygon displays the upper and lower acquired tolerance zone (right triangles) the zone of intrinsic tolerance (rectangle) and total thermal tolerance (entire quadrilateral). Simple Linear regression model of CTmax and CTmin also show the lowest and highest CTmax and CTmin, based on extrapolated regression lines with upper and lower acclimation temperatures CTmax and CTmin, respectively. CTmax = Critical thermal maxima and CTmin = Critical thermal minima.

Fig. 1b. Thermal tolerance polygon created by using CTmax and CTmin data. It shows the thermal tolerance zone of CR hybrid and RC hybrid acclimated to four different temperatures (22, 26, 30 and 34 °C). Polygon displays the upper and lower acquired tolerance zone (right triangles) the zone of intrinsic tolerance (rectangle) and total thermal tolerance (entire quadrilateral). Simple Linear regression model of CTmax and CTmin also show the lowest and highest CTmax and CTmin, based on extrapolated regression lines with upper and lower acclimation temperatures CTmax and CTmin, respectively. CR = *C. catla*♀×*L. rohita*♂ hybrid; RC = *C. catla*♂ ×*L. rohita*♀ hybrid; CTmax = Critical thermal maxima and CTmin = Critical thermal minima.

interspecific hybrids) tested over four fixed temperatures (22–34 °C), thermal tolerance polygon, indicated a significant difference in total, intrinsic and acquire thermal tolerance zones. The results of higher thermal tolerance polygon area of *L. rohita* (360.41°C^2) as compared to *C.catla* (316.98°C^2) is in accord to other reports (Chatterjee et al., 2004; Das et al., 2004) and indicating species specific difference. It is well documented that *C. catla* is a surface feeder, least temperature tolerant species than *L. rohita,* which feed at column or middle layer of pond due to which they have more tendency to adapt adverse conditions (Das et al., 2004).

In the present findings, a significant difference in total as well

Table 2
Putative markers used for Hsp70 and Hsp90 expression study.

Target genes		Gene sequence 5′ to 3′	Amplicon Size (bp)	Accession No
Hsp90	F	GGCAAGGACCTGAAGATC	124	KM:091924.1
	R	CAGACTTGGCGATGGTAC		
Hsp70	F	CAAGAGGAAGCACAAGAAGG	162	KM:369886.1
	R	CTGGTGATGGACGAGTAGAAG		
18-s	F	CGGACACGGAAAGGATTGAC	84	KC:915025.1
	R	CGCTCCACCAACTAAGAACG		

Table 3
Effect of temperatures on immunity and Lipid peroxidation of *L. rohita, C.catla* their intergeneric hybrids.

	L. rohita	*C. catla*	CR hybrids	RC hybrids
Lysozyme (µg/mL)				
12°C	2.06 ± 0.18^{Cbc}	1.61 ± 0.22^{Bc}	2.83 ± 0.19^{Bb}	4.05 ± 0.43^{Ba}
27°C	2.91 ± 0.25^{Bb}	1.72 ± 0.29^{Bc}	3.50 ± 0.19^{ABab}	3.94 ± 0.39^{Ba}
37°C	4.51 ± 0.50^{Aab}	2.63 ± 0.58^{Ab}	4.19 ± 0.42^{Aa}	4.83 ± 0.42^{Aa}
Respiratory burst activity				
12°C	0.34 ± 0.09^{Ba}	0.25 ± 0.04^{Ba}	0.40 ± 0.07^{Ba}	0.45 ± 0.10^{Ca}
27°C	0.41 ± 0.08^{Abc}	0.33 ± 0.05^{ABc}	0.52 ± 0.05^{Bb}	0.70 ± 0.06^{Ba}
37°C	0.58 ± 0.04^{Abc}	0.43 ± 0.06^{Ac}	0.76 ± 0.07^{Ab}	0.96 ± 0.07^{Aa}
LPO (µmol of malondialdehyde/mg protein				
12°C	3.07 ± 0.14^{Bc}	4.94 ± 0.15^{Ba}	4.01 ± 0.13^{Bb}	2.10 ± 0.13^{Bd}
27°C	0.27 ± 0.10^{Cb}	0.45 ± 0.03^{Ca}	0.27 ± 0.03^{Cb}	0.23 ± 0.01^{Cb}
37°C	6.01 ± 0.46^{Ab}	8.38 ± 0.43^{Aa}	7.89 ± 0.37^{Aa}	5.49 ± 0.47^{Ab}

Data presented as Mean ± SE (n = 9). ANOVA followed by LSD show comparisons between populations at same temperature and within population at different temperature. Means sharing different lowercase superscripts within row indicate significant differences between populations, while uppercase superscripts in same column show significant difference at ($P < 0.05$) within population. CR = C. *catla*♀×*L. rohita*♂ hybrid, RC = *C. catla*♂ ×*L. rohita*♀ hybrid.

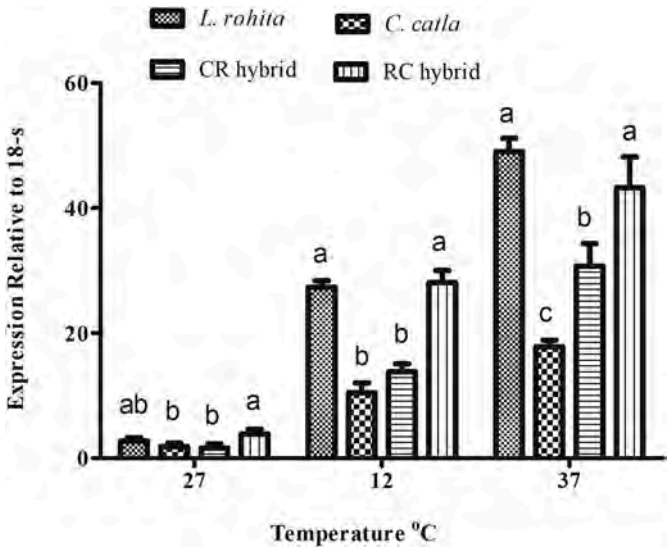

Fig. 2. Expression of Hsp70 gene in the hepatopancreas at different temperatures (12, 27 and 37 °C). Bars shows the values as Mean ± SE, n = 9. ANOVA followed by LSD post hoc test represent comparisons between populations at different temperatures. Different lowercase superscripts on bars are showing significant difference ($P < 0.05$) between populations. CR = C. *catla*♀×*L. rohita*♂, RC = *C. catla*♂ ×*L. rohita*♀.

intrinsic and acquired thermal polygon area among RC and CR hybrids as well between hybrids and parent species indicating the appearance of phenotypic variation. About 11.52% and 5.39% greater polygon area of

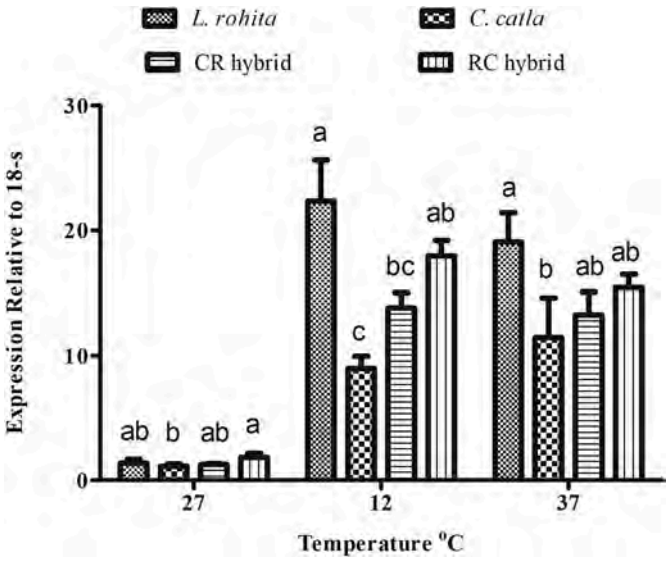

Fig. 3. Expression of Hsp90 gene in the hepatopancreas at different temperatures (12, 27 and 37 °C). Bars shows the values as Mean ± SE, n = 9. ANOVA followed by LSD post hoc test represent comparisons between populations at different temperatures. Different lower-cases superscripts on bars are showing significant difference ($P < 0.05$) between populations.

RC and CR hybrids respectively in contrast to *C. catla,* reveals more temperature tolerant of both hybrids, especially RC hybrid as compared to *C. catla*. However, in contrast to *L. rohita*, RC and CR hybrid displayed 2.1% and 8.1% less thermal polygon zone, indicating low performance However, close observation of the data revealed less acclamatory response of CR hybrid with respect to *L. rohita* in upper temperature tolerance, but greater in lower acquire temperature tolerance. To the best of our knowledge, thermal tolerance of intergeneric hybrids of *C. catla* and *L. rohita* is first time reported here; therefore no parallel reports are available to compare our results.

In this experiment, it was also observed that thermal polygons of all our four populations are higher than 273.5°C^2 reported by Chatterjee et al. (2004) for *L. rohita*, over the acclimation range 25–35 °C, but much smaller than 744.8°C^2 reported by Das et al. (2004) in *L. rohita*, over the acclimation temperature range of 12–40 °C. The variability in the results might be related to differences in acclimation temperature, because the total thermal polygon area is dependent on the acclimation temperatures, used in the trial (Das et al., 2005). It is well documented that first generation hybrids (F1 hybrids) contain a genetic combination of both parental species, which could result in new and better ontogenetic trajectories (Corse et al., 2012). But the results in both reciprocal hybrids were found intermediate (352.85°C^2 for RC and 333.47°C^{2}for CR) between the two pure species, lower than *L. rohita* and higher than *C. catla*.

Due to heterosis, F1 hybrids often show better thermal tolerance as compared to parental lines (Willett, 2011). Conversely, our results indicated that hybridization between *L. rohita*and *C. catla* led to intermediate thermal tolerance. Both hybrids showed relatively low-parent heterosis for this trait, approximately 11% for RC hybrid and 5% for CR, which are less than 20%.This result may reflect that there was no effect of heterosis on thermal tolerance-related trait. It may be due to a very small genetic distance between *L. rohita* and *C. catla* (Dan et al., 2014). It is well documented that genetic distance among species represents strength of heterosis (Li et al., 2012). Like our results, many investigators reported intermediate thermal tolerance in the hybrids of grass carp and bighead carp (Bettoli et al., 1985), Northern and Florida largemouth Bass reciprocal hybrid (Fields et al., 1987). Comparing the two hybrids, RC was found more tolerant to temperature than CR which may be due to maternal effect of *L. rohita*. According to Martins et al. (2019) female parent contribute more significantly in the transmission

of thermal traits.

Temperature shift beyond the optimum range triggers a stress response and modulate the immune response (Dominguez et al., 2004). Here besides determining CTmax and CTmin at various acclimation temperatures, we also assessed the impact of temperature below or above the optimum temperature, on the physiological response of pure species and their intergeneric hybrids and observed variable results. This information is valuable because physiological changes reflect the adaptability of fish to change in water temperature. Our results are in accord to literature where fish showed species specific thermal tolerance and demonstrated different physiological response to increase or decrease in temperature (Abram et al., 2017; Balta et al., 2017).

Heat shock protein Hsp70 and Hsp90 are the potential biomarkers to understand the response of fish against thermal assault. It provides protection to the internal cellular organization and regulates vital cellular functions (Richter et al., 2010; Sharma et al., 2017). Here the up-regulation of Hsp70 and Hsp90 genes in the hepatopancreas of all populations exposed to high (37 °C) and lower (12 °C) temperature indicated the activation of protection mechanism. Like our results many other investigators also reported upregulation of Hsp70and Hsp90 in hepatopancreas of other fish species subjected to heat shock e.g. *C. idela* (Wu et al., 2012; Sharma et al., 2017), *Channa striatus* (Purohit et al., 2014) and *Puntius sophore* (Mahanty et al., 2017). It is well documented that species with higher ability to modulate the expression of Hsp show better adaptability to the changing temperature while those lacking this reaction demonstrated reduced abilities (Somero, 2010). Here, many fold higher expression of both Hsp70 and Hsp90 in *L. rohita* and RC hybrid as compared to CR hybrid and *C. catla* may indicate their higher ability to cope the thermal stress.

Furthermore, the increase in the hepatopancreas LPO level at 37 °C and 12 °C as compared to 27 °C (acclimated temperature) in all populations indicated oxidative damage caused by reactive oxygen species. However, lower level of LPO in *L. rohita* and RC hybrids may also indicate their better adaptability. Similar elevated level of LPO in response to heat shock was also reported in *Channa punctatus* (Kaur et al., 2005), *Dicentrarchus labrax* (Vinagre et al., 2012) and *C. catla* (Sharma et al., 2017). The solubility of oxygen is higher at low temperature, therefore most scientists expected inverse relationship between oxidative stress and temperature but here both parents and their intergeneric hybrids showed the lowest LPO activity at optimum/acclimation temperature (27 °C) followed by at low temperature. It seems that oxidative stress is not directly related to temperature. Like our results Vinagre et al. (2012) also reported similar lowest MDA concentration in *D. labrax* at thermal optimum in contrast to temperature beyond the thermal limit.

Temperature fluctuation also affects the fish immune system. Overall enhance immune response to an increase in temperature and suppression at low temperature is well documented (Abram et al., 2017; Larsen et al., 2018). The fish immune response and virulence of pathogens often correlate with water temperature. Generally, temperature beyond the optimal range induced physiological stress and increases the susceptibility of fish to disease by compromising their resistance. Yet, beside abiotic factors, genetic factor also influence the ability of fish to resist infection (Lu et al., 2019). Here the observed increase and decrease in the respiratory burst and serum lysozymes activity at high or low temperature, respectively as compared to acclimated temperature, indicated the activation and slowdown of the of defense mechanism in all populations.

Lysozyme level and respiratory burst activity are the widely used indicators of nonspecific immune system (Saurabh and Sahoo, 2008). Lysozyme provides protection against gram-negative and gram-positive invading bacteria by attacking bacterial cell wall and hydrolyzing peptidoglycan layer. Respiratory burst activity is an indication of inflammation and production of various reactive oxygen species (ROS). It is an important reaction that occurs in phagocytes for degrading the invading organisms (Jian and Wu, 2003). Like our results, Sharma et al. (2017) also reported significant increase and decrease in similar immunological indices in *C. catla* exposed to temperatures higher or below the acclimation temperature. These physiological changes reflect the adaptability of fish to change in water temperature. Here higher levels of respiratory burst and serum lysozyme activity in RC hybrid followed by CR hybrid and *L. rohita* as compared to *C.catla* may indicate the heterosis and better adoptability of hybrids in contrast to parent species especially low parent i.e., *C. catla*.

5. Conclusion

Present study provides the valuable information about the inheritance of thermal tolerance traits in the reciprocal hybrids of *L. rohita* and *C. catla* and their ability to combat the thermal stress as compared to parent species. All populations showed significant differences in CTmin and CTmax values at different acclimation temperatures, which may be linked to their genetic makeup. Results of thermal tolerance assay and expression of Hsp70 and Hsp90 genes, serum lysozyme level, respiratory burst activity and lipid peroxidation level at temperatures, higher or below the acclimation temperature revealed the improved tolerance of both hybrids as compared to *C. catla*. It seems that both hybrids have better adaptability, thus can be advised to manage breeding program and by controlling crosses of *L. rohita* and *C. catla* in each generation produce F1 hybrids, especially RC hybrid for culturing in relatively higher and lower temperature areas.

Ethical approval

The current study was carried out by following the principles presented by the society for prevention of the cruelty to animals (SPCA) of Pakistan. Furthermore, the study was approved (BEC-FBS-QAU2018-16) from the "Bioethical Committee of the Faculty of Biological Sciences, Quaid-i-Azam University, on the use of animals for Scientific Research".

Declaartion of competing interest

The authors have no conflicts of interest to declare.

CRediT authorship contribution statement

Muhammad Ahmad: Formal analysis, Investigation, Methodology, Writing - original draft. **Amina Zuberi:** Conceptualization, Supervision, Validation, Writing - review & editing. **Mashooq Ali:** Resources, Project administration. **Awal Syed:** Visualization, Investigation. **Mahmood ul Hassan Murtaza:** Formal analysis, Writing - original draft. **Abbas Khan:** Software. **Muhammad Kamran:** Data curation, Methodology.

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Appendix A. Supplementary data

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