Effect of Rearing Environment on Hypothalamic-Pituitary-Interrenal (HPI) Axis of Mahseer (*Tor putitora*)

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Effect of Rearing Environment on Hypothalamic-Pituitary-Interrenal (HPI) Axis of Mahseer (*Tor putitora*)

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By

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Islamabad

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CERTIFICATE

This dissertation *''**Effect of Rearing Environment on Hypothalamic-Pituitary-Interrenal (HPI) Axis of Mahseer (Tor putitora)**''* submitted by **Imdad Ullah** is accepted in its present form by the Department of Animal Sciences, Faculty of Biological sciences, Quaid-I-Azam University, Islamabad Pakistan as satisfying the thesis requirement for the degree of Doctor of Philosophy in Fisheries and Aquaculture.

Certificate of Approval

This is to certify that the research work presented in this thesis, entitled **"Effects of Rearing Environment on Hypothalamic-Pituitary-Interrenal (HPI) Axis of Mahseer (*Tor putitora*)"** was conducted by **Mr. Imdad Ullah** Under the supervision of 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 Animal Sciences of Quaid-i-Azam University, Islamabad in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Field of Fisheries and Aquaculture.

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Declaration

I hereby declare that the work presented in the following thesis is my own effort, except where otherwise acknowledged, and that the thesis is my own composition. No part of this thesis has been previously presented for any other degree.

IMDAD ULLAH

IN THE NAME OF ALLAH

THE MOST MERCIFUL

THE MOST BENEFICENT

AND

THE MOST COMPASSIONATE

Dedicated to:

‘‘My loving and caring
Humaira Rehman (Mary)’’

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LIST OF ABBREVIATIONS

Abbreviations	Full Names
®	Registered
°C	Centigrade
µg	Microgram
µl	Microliter
µm	Micrometer
5-HIAA	5-hydroxyindoleacetic acid
5-HT	Serotonin (5-hydroxytryptamine)
cm	Centimeter
DA	Dopamine
dd	Double distilled water
DHBA	2,3-Dihydroxybenzoic acid
DO	Dissolved oxygen
DOPAC	3,4-dihydroxyphenylacetic acid
EIA	Enzyme-linked immunosorbent assay
Ft	Feet
g	Gram
hr	Hour
HVA	Homovanilic acid
mg/dL	Milligram per decilitre
mgL^{-1}	Milligram per litre
min	Minute
ml	Millilitre
mm	Millimetre
mmole	Millimole
mmole/L	Millimole per litre
Ms222	Ethyl 3-aminobenzoate methanesulfonic acid
ng/L	Nanogram per litre
ngg^{-1}	Nanogram per gram
ppm	Part per million
rpm	Rounds per minute
sec	Second
TMB	3, 3, 5, 5-Tetramethylbenzidine

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GENERAL ABSTRACT

Release of hatchery reared fish in the natural environment is one of the most important strategies to replenish the natural stock of endangered fish species mahseer (*Tor putitora*). Most of the reintroduction projects are not providing the desired results, since captive-reared fish may not possess the well-adapted behavioural skills and physiological responses required for survival in the natural environment. Early rearing environment affect the stress sensitivity and shaping the behaviour of fish, while enrichment in the rearing environment, improve the behaviour and physiological responses of fish by modulating HPI axis and dopaminergic and serotonergic systems. Here an attempt has been made to investigate the impact of rearing environment on the physiological stress response and behaviour of endangered fish *Tor putitora*. First, the physiological status of wild caught and captive reared fish was compared by adopting invasive and non-invasive methodology and measured the pre-stress and post stress levels of plasma and water-borne cortisol, blood glucose, whole brain serotonergic activity (5HIAA/5HT ratio), dopaminergic activity (DOPAC/DA and HVA /DA ratios) and Norepinephrine (NE) levels. Life skill activities like exploratory, predatory and anti-predatory behaviour of both populations under laboratory condition were also studied. The captive reared mahseer displayed a typical stress response i.e., low activation of HPI axis and brain monoamenergic (serotonergic and dopaminergic) system and delay recovery period as compared to wild counterpart. Moreover, the wild fish display significantly ($p < 0.05$) more exploratory, predatory and anti-predatory behaviour in comparison to the captive reared fish. However, captive mahseer appeared bolder. In the second part of the study, to test the impact of the enriched rearing environment on hypothalamic–pituitary–interrenal (HPI) axis, brain monoamines system and life skills of fish were devised three different rearing environments (barren, semi-natural and physically enriched) that differed in their levels of complexity and heterogeneity and reared mahseer 15 days old hatchlings up to advanced fry stage in these environments. Similar non-invasive and invasive techniques as used for comparison of wild caught and captive reared fish were followed for evaluation of pre and post stress levels of whole-body and water borne cortisol, blood glucose and brain monoamines as well as the behaviour of fry from three different rearing environments. The pre-stress basal cortisol, blood glucose and brain monoamines serotonergic, dopaminergic and central norepinephrine (NE) levels were higher in fry reared in barren

and semi-natural environment indicating the impact on rearing environment on the stress sensitivity fish. Furthermore, exposure of acute physical stress induced increase in whole-body cortisol, blood glucose, whole-brain serotonergic and dopaminergic activity and Norepinephrine (NE) levels in all three different rearing groups up to 0.75 hr. Although, the peak levels of all stress parameters were observed at 0.75 hrs post stress, but there was a significant difference in the increasing trend and magnitude of cortisol, blood glucose and brain monoamines at peak levels among three rearing groups of mahseer previously reared in different rearing environments. At 0.25 hrs, rapid stress response was observed in semi-natural reared mahseer, compared with barren and physically-enriched reared fish. After 0.75 hrs post stress, the levels of cortisol, blood glucose, brain-monoamines (ratios of 5HIAA/5HT, DOPAC/DA and HVA /DA) and NE in all rearing groups showed a steady decreasing trend and recovered to its basal level after several hours of stress. The recovery time of whole-body cortisol, blood glucose and whole-brain monoamines were significantly higher in barren reared mahseer compared with the other two groups. Moreover, it was also observed that the increased structural complexity during early life significantly affect various behavioural characteristics of the fish. Exploratory, predatory and anti-predatory behaviours were significantly ($p < 0.05$) more pronounced in fry reared in physically enriched and semi-natural environment than barren environment. The results of the present study specify the role of rearing environment in shaping the stress response and life skill activities of fish and suggest an improvement in the hatchery rearing environment to reduce the physiological and behavioural variations among wild and captive reared counterpart. We further illustrate that the increased structural complexity, i.e., physical enrichment in the early rearing environment significantly modulates various physiological stress coping mechanisms and life skill behaviour of mahseer. These outcomes have important implications for a possible way of improving the outcomes of restocking programs of endangered fish species by modifying conventional hatchery-rearing environments.

GENRAL INTRODUCTION

Mahseer (*Tor putitora*) national fish of Pakistan, is among the most important freshwater fish species of the Indian sub-continent (Everard and Kataria, 2011; Gupta et al., 2014; Bhatt and Pandit, 2016). It is a large cyprinid fish that is considered as cultural icons of economic, recreational and conservation interest in many Asian countries, including Pakistan and more importantly serve as a 'freshwater flagship'. The Himalayan or Golden mahseer (*Tor putitora*) is acclaimed as world famous, outstanding game (hardest fighting fish) and food fish (Everard and Kataria, 2011; Gupta et al., 2014) that provide unparalleled recreation to anglers from all over the world (Gupta et al., 2014; Bhatt and Pandit, 2016). It is among the mega fishes' of the world (Bhatt et al., 2000; Stone, 2007), attains nearly 3 m length and over 54 kg weight (Everard and Kataria., 2011; Nautiyal et al., 2008). As a food fish, it is highly esteemed and fetches the highest market price in the Asian countries. In commercial fisheries it occupies an important position for its good quality and high market value, while for fishermen; mahseer is of considerable importance because of its large size.

Mahseer is endemic to Asia with a wide distribution spanning from Afghanistan, Pakistan, India, Sri Lanka, Nepal, Bhutan, Myanmar, Thailand, China, Laos, Cambodia, Vietnam, Indonesia and Malaysia (Menon, 1992; De Silva et al., 2004; Mohindra et al., 2007; Nautiyal et al., 2008; IUCN, 2016). It is distributed in foothills of Himalayan region and has a wide distribution in sub-continent (Talwar and Jhingran, 1992; Bakawale and Kanhere, 2013). Earlier studies indicated the presence of mahseer in most of the rivers of Pakistan (Mirza and Khan, 1994; Mirza et al., 1994; Zafar et al., 2001), but recent studies reported that this population reduced from most parts of the country (Shafi et al., 2016) and the only size able and stable population that remains is in the Mahseer National Park (Poonch river), Azad Jammu and Kashmir, Pakistan (Rafiq and Khan, 2012).

T. putitora is mostly rheophilic in nature, inhabiting hill streams having rocks / stones substrate (Nautiyal, 2014). The foothill stretches of the Himalayan Rivers are reported to be the stationary or feeding grounds of the golden mahseer. These habitats have large volumes of water and the river bed is mostly covered with sand, silt and small boulders (Bhatt et al., 2004). The physico-chemical nature of the feeding

grounds is characterized by water temperature in the range of 14–22ºC and an alkaline (pH>7). Dissolved oxygen in these habitats varies from 5.2 to 12.9 mgL^{-1} (Bhatt et al., 2004). It mostly inhabits semi-cold natural running waters (Shrestha et al., 1990; Talwar and Jhingran, 1992; Bhattand Pandit, 2016), while reservoirs with clear water, rocky beds, submerged aquatic plants, filamentous algae, molluscs and insects provide a suitable environment for its existence (Desai, 2003). Similarly, they are also found in streams hardly above sea level and at an altitude of 2000 m above sea level. The occurrence and distribution of mahseer are controlled by the prevailing water temperature of the streams and not by the altitude (Jhingran and Sehgal,1978)

The food of *T. putitora* comprises insects, algae, macrophytes, rotifers, small fish, crustaceans etc.(Desi, 2003; Bhatt and Pandit, 2016). Numerous studies have indicated that the species feeding habits change with increasing size. At early stages of its development, it is carnivorous (Froese and Pauly, 1999) and later on diverts to omnivore when approaches to juvenile stage. Sometimes it becomes an opportunistic feeder and also feeds on larvae, small mollusks and algal coating on rocks (Shrestha, 1997). Examination of guts of fry of *T. putitora*, for example, revealed diatoms as the chief food item (Bhatt and Pandit, 2016), while in fingerlings and juveniles, researchers have found 81.4 % insect material and 15.9 % plant material in their guts and categorized this stage of fish as insectivorous (Nautiyal and Lal, 1984).The anatomical adaptations of the alimentary canal system also confirm that mahseer are omnivorous that feed on plants, insects, shrimp and mollusks (Talwar and Jhingran, 1992; Froese and Pauly, 1999; Desi, 2003; Bhatt and Pandit, 2016).

Mahseer is potamodromous (migrating within freshwaters), perform seasonal migrations within a short distance mainly for feeding and breeding. The limit of such migrations is determined by water temperature and floods. Mahseer prefer rocky pools and cooler temperature, moving up and downstream, depending upon the flood conditions (Nautiyal, 1994). Johnsingh et al. (2006) suggested that the mahseer moves upstream in search of suitable spawning grounds, while turbid waters and higher water temperature are considered to be the stimuli for migration for breeding in the rain-fed hill streams (Nautiyal, 1994; Bhatt et al., 2004). Hence, migratory habit of mahseer is attributed to the changes in water temperature and to seek more congenial

surroundings during the monsoon (Shrestha, 2002; Bhatt et al. 2004; Johnsingh et al., 2006; Nautiyal, 1994)

Generally, the spawning grounds of the fish are characterized by river beds with large boulders, pebbles and gravel with water temperature varying from 11 to 30.5˚ C, alkaline pH and dissolved oxygen concentration in the range of 6.4–11 mg L^{-1} (Bhatt et al., 2004; Joshi, 1994). In Pakistan the natural spawning areas of this species are River Korang near Islamabad, Pakistan (Zafar et al., 2001), mahseer National park (River Poonch) Azad Jammu and Kashmir and allied rainy streams, River Swat and Kalpani River, Mardan (Khyber Pakhtunkhwa), Harro River Attock and River Indus. Previous reports suggested that the species spawns twice in a year, during April–May and July–October (Malik, 2011). In Pakistan, the spawning season of mahseer ranges from early March to mid May and August - October in captivity (personal observations). However, Hussain and Mazid (2001) reported that in Bangladesh, the species spawn during early November to late January. Absolute fecundity of *T. Putitora* is very low i.e., between 8000 and 12,000 eggs/kg body weight of female (Mahata et al., 1995; Hussain and Hossain, 1999), compare to other carp species.

Globally, one third of all freshwater fishes, including mahseer are threatened with extinction (Dudgeon, 2012 ; Gray, 2013). Various factors, including habitat loss, overexploitation and biological invasions are contributing significantly in destruction of various species (Dudgeon, 2012; Gozlan et al., 2005). Like other threaten species, many mahseer (*Tor putitora*) populations have experienced severe declines across their natural range because of anthropogenic disturbances, including damming on rivers, deforestation, pollution, and overexploitation (Lakra et al., 2010; Nautiyal, 2011, 2014; Pandit and Grumbine, 2012; Khajuria et al., 2013; Gupta et al., 2014, 2015; Sharma et al., 2015). Many current literatures have reported that this fish is extremely vulnerable and threatened in the most part of the Asia and Trans-Himalayan region of sub-continents.

Over the last two decades, initiation of hydropower projects to meet the energy requirement of growing population in Himalayan region resulting change in landscape and habitat destruction (Grumbine and Pandit, 2013; Pandit et al., 2014).

Construction of multiple dams on the Himalayan rivers cause changes in the quantity, quality and regime of water flow in the downstream sections of rivers that results in habitat fragmentation of migratory species, habitat degradation, submergence of large terrestrial and river bed areas (Everard and Kataria, 2011; Gupta et al., 2015; Quinones et al., 2015). The major anticipated impacts of dams on the Himalayan rivers are decreased water flow, rise in channel water temperature, diurnal flow variation, low turbidity of downstream waters as well as the interruption in the longitudinal connectivity (Bunn and Arthington, 2002). All these factors are responsible to hinder fish migration that is required for reproduction and increase mortality by higher predator attacks (Abrahams and Kattenfeld, 1997; Bunn and Arthington, 2002; Bhatt et al., 2000). Beside these, many other factors like destruction of breeding grounds, indiscriminate fishing of broodstock and juveniles, wanton (illegal) killing of juveniles and brood fishes through poisoning or dynamiting (khan and Sinha, 2000), use of small mesh nets, explosive, plant-derived toxins, poisons and electro fishing by poachers, industrial and human pollution etc are also contributing in declining the population of the *T. putitora* in Himalayan region. Moreover, life history traits migratory behavior, low fecundity (William et al., 2005) and delayed sexual maturity (Nautiyal and Singh, 1989) also combined with numerous external threats for declining its population (William et al., 2005). Obstruction in migratory route due to construction of dams cause gathering of migratory fish in particular area and make them vulnerable to predators and exploitation by humans (Ogale, 2002).

Depletion of mahseer populations has been reported from various parts of India (Desai, 1994; Laskar et al., 2013; Gupta et al., 2014; Bhatt and Pandit, 2015), Pakistan (Mirza, 1994; Mirza et al., 1994; Yaqoob, 2002), Nepal (Shrestha, 2002), Turkey (Balik, 1995), Papua New Guinea (Coates, 1991) and Bangladesh (Hussain and Mazid, 2001). According to the red list of IUCN, mahseer is identified as a critically endangered species (IUCN, 2015, 2016), had been declined about 50 % in the past and may decline more by about 80 % in future, specially because of the regulation of the rivers it inhabits (Sharma et al., 2015; IUCN, 2016). Conservation of economically important and endangered *T. puitora* is therefore, a serious challenge and its alarming natural population reflecting the need of serious efforts for its rehabilitation and conservation. Many scientists have suggested special attention for the protection of mahseer from extinction (Yaqoob, 2002; Nautiyal et al., 2008;

Everard and Kataria, 2011; Naeem et al., 2011; Arora and Julka, 2013; Sati et al., 2013; Khajuria et al., 2013; Laskar et al., 2013; Gupta et al., 2014; Ali et al., 2014; Bhatt and Pandit, 2015).

Realizing ecological and economic importance of mahseer, particularly in the Himalayan regions, several attempts have been made to propagate and rehabilitate the species in rivers and lakes by initiating artificial breeding and restocking programs (Pandey et al., 1998). However, the outputs of restocking programs are not encouraging and the natural populations of mahseer are still showing continuous decline. Like other countries, Pakistan has also taken step for the conservation of this species and established hatcheries in different provinces (Punjab and Khyber Pakhtunkhwa) and initiated the artificial propagation program. Moreover, for replenishment of natural stock, restocking program was also initiated that involve the release of artificially propagated hatchery reared fish in natural bodies (rivers, streams, lakes). However, in spite of all efforts, the results are also not encouraging and captures of this species from natural reservoirs are continuously declining (Personal observation and communication with Punjab Fisheries). It seems that hatchery reared fish in the natural environment underwent mortality may be due to behavior deficiencies or atypical physiological responses. It appears that for the conservation and rehabilitation of this species, development of a suitable technology in contrast to conventional methodology for breeding, rearing and nursing of fry and fingerlings is required (Islam, 2002; Rahman, 2003).

Restocking programs has been commonly used in attempts to counter the effects of over-fishing, environmental degradation and recruitment failures. However, restocking and conservation programs are controversial with respect to hatchery reared populations as well as to the ability of fish to maintain successfully in their natural population (Olla et al., 1998; Salvanes, 2001; Myers et al., 2004). Generally releasing programs rely upon the assumption that captive-bred or translocated animals can rapidly adjust to a wide diversity of novel challenges upon release. However, many captive breeding programs fail to raise individuals with natural behaviour, thus showing high mortalities upon release (Araki et al., 2008). Recent experiments on fish show that the lack of stimulus variation in captive rearing conditions influence the

phenotype at many different levels, ranging from physiology, neurology to behaviour (Olla et al., 1998; Huntingford, 2004; Brännäs and Johnsson, 2008).

The current failure of stock enhancement projects might be related to inadequate rearing environment at hatcheries (Leber, 2013; Lee and Berejikian, 2008), which often generated fish unable to survive in the wild (Lorenzen et al., 2010; Salvanes and Braithwaite, 2006). Standard hatchery conditions typically consist of plain impoverished environments, often designed to sustain good physical health. Nevertheless, such structure-less/featureless environments can severely restrict the natural behavioural and physiology of animals, and may further compromise with welfare of fish (Brännäs and Johnsson, 2008; Huntingford, 2004).It has been previously observed that upon release hatchery-reared fish have lower survival rates and provide lower returns to anglers than wild fish (Ebner and Thiem, 2007; Daniels and Watanabe, 2010; El Balaa and Blouin-Demers, 2011). Poor survival greatly reduces the efficiency of hatchery stocks to supplement wild population (Maynard et al., 1995; Olla et al., 1998). It is reported that in most instances mortality is highest during and immediately after release into the wild (Heggberget et al., 1992; Olla et al., 1998). Hatchery selection favors fish that are well adapted to captivity, but maladapted to the wild (Christie et al., 2012), leading to differences in behavior, physiology and survival between wild and hatchery stocks.

Captive animals exist in a very different dynamic environment as compare to natural one in term of resource availability, rearing densities and dangers involved (Boersma et al., 2008; Epp and Gabor, 2008; Brockmark et al., 2010), and encounter experiences different from their free-living counterparts (Price, 1999; Huntingford, 2004). In captivity, animals are protected from predation, competition and disease causing organisms. Animals are habituated to captive environments due to human intervenes, limited spaces, constant availability of food and water and protection from predators (Price, 1999). Extending such a life style to several generations as well as intentional or intentional selection, allow individuals genetically predisposed to suit these conditions (Price, 1999), like aggression to compete for food, or poor predation-avoidance abilities (Stunz and Minello, 2001; Huntingford, 2004). Captive conditions, as well as the lack of life training experiences present in the wild, often produce individuals unfit for wild environments (McPhee, 2003; Mathews et al.,

2005; Jule et al., 2008). Hatchery reared fish upon release into wild feel difficulty to capture live prey (McNeil, 1991; Olla et al., 1998) in high predation pressure and high mortality may be due to maladaptive behavior and physiology (Huntingford, 2004; Fuiman et al., 2010; Brown et al., 2016).

Hatchery reared fish in semi intensive and intensive aquaculture facilities face several potential environmental, social, and husbandry stressors, including limited space, high density, and aggressive interaction with conspecifics, handling and confinement stress. To cope such situations, fish harmonize physiological and behavioural responses and release major stress hormones like corticosteroids and catecholamines (Schreck, 2000; Barton, 2002; Pankhurst, 2011; Wendelaar Bonga, 1997). Cortisol, is the frequently use indicator of acute and long-term stress in fish that is released by the hypothalamic–pituitary–interrenal axis (HPI-axis) (Barton, 2002; Martinez-Porchas et al., 2009). The elevated level of cortisol can lead to energetic costs (Leal et al., 2011), modulate metabolic process by effecting energy-demanding processes, like growth (Bernier et al., 2003), reproductive process (Fitzpatrick et al., 2012; Schreck, 2010), immune function (Tort, 2011) and neurogenesis (Sorensen et al., 2011). The stress responses of captive bred fish could be effected by adaptation of animals to domestic environment or though intentional and unintentional artificial selection at the time of breeding.

Wild fish may respond differently to stressors because of genetic or environmental differences (Clements and Hicks, 2002), e.g. domesticated fish generally show increase body weight , higher growth and reproduction rates compare with their wild counterparts (Wright et al., 2006). In birds and mammals, domestication lead to lower reactivity of the stress axes (Künzl et al., 2003; Ericsson et 2014; Fallahsharoudi et al., 2015). Similar variation can be seen in wild and domestic strains of brown trout (*Salmo trutta*) (Lepage et al., 2000), rainbow trout (*Oncorhynchus mykiss*) (Jentoft et al., 2005), fighting fish (*Betta splendens*) (Verbeek et al., 2008), and rainbowfish (*Melanoteania duboulayi*) (Zuberi et al., 2011).

Fish either in captivity or in natural environment, faces wide range of stressors around the clock (Galhardo and Oliveira, 2009) and show physiological and behavioural responses. The physiological stress response are very well studied in

many teleosts (Barton and Iwama, 1991; Wendelaar Bonga, 1997; Barton, 2002; Ellis et al., 2004; Zuberi et al., 2011) and appears as biphasic with an initial short latency elevation of plasma catecholamines from chromaffin tissue of kidney, followed by prolonged elevation in plasma cortisol levels from interrenal tissue (Wendelaar Bonga, 1997; Pankhurst, 2011). Like other vertebrates, corticosteroid hormones concentration in blood is a major index of stress in fish, and activation of the hypothalamus-pituitary-interrenal (HPI) axis is responsible for increase their concentration (Tsalafouta et al., 2014; Pijanowski., 2014).

Under stress, the preoptic area of the brains of many fish species like carp *Cyprinus carpio* (Flik et al., 2006), masou salmon *O. masou* (Westring et al., 2008), rainbow trout *Oncorhynchus mykiss* (Craig et al., 2005; Bernier and Craig, 2005; Doyon et al., 2006) showed improve expression and synthesis of corticotropin releasing factor (CRF), which in turn regulated by cortisol via negative feedback mechanism (Bernier et al., 1999; Doyon et al., 2006). It is suggested that corticosteroids in fish usually exert their actions through classical genomic and non-genomic action (Borski et al., 2001). CRF stimulate the release of ACTH, α-melanocyte stimulating hormone (α-MSH) and β-endorphin by of stimulating the synthesis and cleavage of pro-opiomelanocortin, precursor of these hormones (Sumpter, 1997). CRF besides stimulating the release of ACTH also has neuromodulatory and behavioural effects. Generally, it stimulates the locomotory activity (Clements et al., 2002; Lowry and Moore, 2006), and suppress the appetite and feeding behaviour in a range of species (Bernier and Peter, 2001; Bernier and Craig, 2005; Bernier, 2006).

The time course production of cortisol in response to stress is variable among species but variation in response latency (time to identify significant increase) is, in minutes rather than hours, e.g. response times of striped bass (*Morone saxatilis*) *w*as as short as 2.5 min (Tamasso et al., 1996) while in the sea raven (*Hemitripterus americanus*) as long as 120 mins (Vijayan and Moon, 1994). It is reported that response latencies are independent of temperature but related to the lifestyle of species. Most active species showed quick post-stress increases in cortisol as compared to sedentary species (Vijayan and Moon, 1994; Wright et al., 2007).

Magnitude of the corticosteroid response also shows considerable variability among species and appear as individual characteristic which is stable over time, with a moderate to high degree of heritability (Pottinger et al., 1994; Fevolden et al., 1999). Generally, the peak values lie between 30-300 ng mL^{-1} but within species variation obseve according to severity of stressor and the duration of exposure (Barton, 2002). In contrast to attain peak cortisol level , the return of plasma cortisol to basal value (recovery from stress) taker lomger time and occur in hours rather than minutes. Although quick falls in plasma cortisol may reveal recovery but reflect the desensitisation of the HPI axis because of continued exposure to stressors (Cyr and Romero, 2009). The recovery from acute stress may be as short as 2-6 hr (Robertson et al., 1988; Young and Cech, 1993) or as long as 24-48 hr (Vijayan and Moon, 1994; Barnett and Pankhurst, 1998).

In addition to cortisol, glucose and lactate levels also increase under stress. Generally, in initial stage catecholamine-mediated glycogenolysis is responsible for the increase in plasma glucose but at later stages, elevation is due to cortisol-mediated gluconeogenesis (reviewed in Begg and Pankhurst, 2004; Mommen et al., 1999). Rises in plasma glucose vary in different fish species and depend on the capacity to store glycogen in lever (Pottinger et al., 2002; Wright et al., 2007). Moreover, after acute stress, plasma glucose profile (to attain peak level and return back to basal level) show variability with cortisol profile (Pottinger, 1998; Flodmark et al., 2002).

In addition to HPI axis, brain monoaminergic systems (dopaminergic and serotonergic systems) also regulating the stress rsponses, independently or by regulating the HPI axis (Chaouloff, 1993; Winberg and Nilsson, 1993). In vertebrates including fish, common control mechanisms in the brain regulate the behavioral and physiological stress responses while monoamine neurotransmitters serotonin (5-hydroxytryptamine, 5-HT), dopamine (DA), and norepinephrine (NE) are synchronizing the action (Winberg and Nilsson, 1993; Winberg et al., 2001; Øverli et al., 1999; Höglund et al., 2002; Lepage et al., 2003; Clements et al., 2003; Larson et al., 2003; Perreault et al., 2003). In fish like other vertebrates brain serotonergic activity indicated by 5-hydroxyindoleacetic acid (5-HIAA, the major serotonin metabolite) 5-HIAA to serotonin (5-hydroxytryptamine, 5-HT) ratio increase quickly under stress (Winberg and Nilsson, 1993). Similarly, acute stress also show a rapid

activation of brain dopaminergic (DA) and norepinephric systems in rainbow trout (Øverli et al., 1999).

Several studies observed plasma cortisol concentrations correlation with the brain 5-HIAA/5-HT ratios and, suggesting the involvement of brain 5-HT, in the regulation HPI axis (Winberg and Lepage, 1998; Øverli et al., 1999; Höglund et al., 2000). It is believed that 5-HT stimulate the hypothalamic–pituitary–adrenal (HPA) axis in mammals (Heisler et al., 2007), as well as the hypothalamic–pituitary–interrenal axis (HPI axis) in fish (Winberg et al., 1997; Øverli et al., 2000; Höglund et al., 2000). However, the involvement of central DA in the regulation of the HPA axis are still controversial and in mammals, central DA has been suggested to act stimulatory, inhibitory or no role in the regulation of the HPA axis (Brambilla et al., 2000; Sullivan and Dufresne, 2006).Winberg et al. (1997) observed dose dependent elevated plasma cortisol after treatment of rainbow trout (*Oncorhynchus mykiss*) with a potent 5-HT1A receptor agonist, 8-hydroxy-2-(di-n-propylamino)-tetralin (8-OH-DPAT). It is documented that 5-HT precursors or 5-HT receptor agonists elevates plasma glucocorticoid levels in mammals, while inhabitors show opposite effects (reduce the levels) (Winberg et al., 1997; Dinan, 1996).

Noradrenaline also play role in triggering the release of CRF, leading to the activation of the HPA axis in mammals (Dunn et al., 2004). Fish brain monoamine activation related to social interactions, especially social status and aggressive behaviour (Summers et al., 2005; Blanchard et al., 2001; Johnsson et al., 2006). For instance increase serotonergic activity in rainbow trout *Oncorhynchus mykiss* was observed at higher stocking density (Laursen et al., 2013), while increase dopaminergic activity in white seabream (*Diplodus sargus)* (Papoutsoglou et al., 2006) was associated with high social stress.

Early rearing environments and experiences play significant role to shape an animal behaviour (Huntingford, 2004; Liedtke et al., 2015). Like many other vertebrates including old field mice (McPhee, 2003) and mussels (Hoftyzer et al., 2008), captive environments promote domesticated behavior in wild fish (Kohane et al., 1988; Kelley et al., 2006; Lee and Berejikian, 2008) such as incorporate change in predator recognition (Kelley and Magurran, 2003; Brown, 2003; de Azevedo and Young, 2006), foraging, and reproductive behaviour (Kelley et al, 2006). In fish, captive bred individuals seek less refuge (Kelley et al., 2006) show higher mortality

due to predation (Jepsen et al., 2000; Kekäläinen et al., 2008) and low resistance to disease than wild counterpart after release to the wild (Johnsen and Jensen, 1991).

Hatchery-reared animals may lack the necessary behavioral skills to forage natural prey, exhibit deleterious morphologic characteristics (Purcell, 2002) or display behaviors that increase their vulnerability to predators (Kellison et al., 2000; Castro and Cobb, 2005). At hatchery, fish have intentionally and unintentionally been selected for high growth rate, which lately has been connected to a shorter memory duration, thus may simply forget fast what they have learned (Brown et al., 2011). In semi-intensive and intensive culture systems, fish are typically grown in static, featureless environments with unnaturally high densities. They are provided an excess of pellet food, thus preventing them to learn how to capture natural live prey. However, wild fish live in complex environments and learn by experience how to capture and handle various live prey types (Sundström and Johnsson, 2001). Fish reared for conservation purpose, behaviour adapted to natural environments are critical for obtaining stocking effectiveness (Brown and Day, 2002; Salvanes and Braithwaite, 2006). Hence, concern regarding the poor performance of hatchery reared fish following release (Salvanes and Braithwaite, 2006; Le Vay et al., 2007), greatly reducing the effectiveness of restocking program

Hatchery-reared animals may simply be ill-equipped for the transition from hatchery conditions to the natural environment and suffer high rates of mortality (Brown and Day, 2002; Huntingford, 2004; Bell et al., 2005; Le Vay et al., 2007). Behavior can be among the first traits affected by domestication and differences in behavior between hatchery reared and wild fish are well documented (Olla et al., 1998; Alvarez and Nicieza, 2003; Lee and Berejikian, 2008). Domestication can promote phenotypic traits that are different from their wild counterparts (Price, 1984; Larson and Fuller, 2014).

Hatchery reared fish are physiologically and behaviorally deficient to cope the challenges of natural environment, thus has led many questions about the validity of stock enhancement from hatchery reared fish sources (Brown and Day, 2002; Huntingford, 2004). Previous studies have demonstrated that behavioural deficiencies or maladaptive behaviour of fish can be reduced by proper pre-release conditioning of

hatchery-reared fish to a variety of stimuli (Griffin et al., 2000; Alvarez and Nicieza, 2003; Kelley and Magurran, 2003). Life-skills training, social learning protocols and environmental enrichment (Brown and Day, 2002) can be adopted to enhance the post-release survivorship of fish (Brown and Laland , 2001). It has been suggested by studying several fish that enriched captive environments can promote behavioural flexibility (Braithwaite and Salvanes, 2005), foraging abilities (Brown et al., 2003; Strand et al., 2010; Rodewald et al., 2011) influence social interactions (Berejikian et al., 2000; Salvanes and Braithwaite, 2005) and reduce anxiety (Maximino et al., 2010). Standard laboratory housing conditions typically consist of plain impoverished environments, often designed to standardize behaviour between different experimental groups and maintain good physical health (Olssonand Dahlborn, 2002). However, such environments can severely restrict the natural behavioural repertoire of animals, and hence may compromise their welfare if the animal is highly motivated to carry out particular behaviours (Dawkins, 1998).

Recently, environmental enrichment appears as a mitigating strategy, increasingly used by hatchery managers to reduce stress and maximize psychological and physiological well-being of captive animals by identifying and providing the appropriate environmental stimuli (Shepherdson, 2002; Gerber et al., 2015). It is generally accepted that structural enrichment and complexity in the rearing environment, improve animal welfare and living performance in captivity (Shepherdson, 2012; Young, 2013). Increased structural complexity in the rearing environment resulted in improved growth (Batzina, 2014), survival (Coulibaly et al., 2007) and foraging behaviour (Strand et al., 2010) in several hatchery reared fish species. The conservation of endangered species programs, especially those which produce individuals for the reintroduction into wild, can be benefited by environmental enrichment by promoting the development of healthy, reproductively successful, and behaviorally competent animals (Swaisgood et al., 2002).

Extensive literature is available on fish indicating that more complex rearing environments promote the development of fish brains (Kishlinger and Nevitt, 2006; Näslund et al., 2012), cognitive abilities (Brown et al., 2003; Kotrschal and Taborsky, 2010; Strand et al., 2010), behaviour (Braithwaite and Salvanes, 2005; Salvanes and Braithwaite, 2005; Salvanes et al., 2007; Moberg et al., 2011; Roberts et al., 2011)

and survival in the wild (Maynard et al., 1996). Social and physical enrichment change the behaviour and physiology of fish and often consider beneficial for the captive animals (Balcombe, 2005). It can be use to decrease fear and aggression (Reinhardt, 2004) and improve physiological stress responses (Fox et al., 2006). For example, female, group housed rats were less stressed than those housed in isolation (Sharp et al., 2003). Previous behavioural observations (Baker, 1997; Swaisgood et al., 2002) and measurement of glucocorticoid hormonal values (Belz et al., 2003) have suggested that enriched environments can reduce adrenocortical activity in captive animals.

Structural enrichment reduced the basal level of stress hormone cortisol concentrations and improve the antipredator behaviour in hatchery-reared Atlantic salmon (Näslund et al., 2013). There are several observations demonstrating the positive effects of environmental enrichment on welfare and survival of many fish species (see review Näslund and Johnsson, 2014). Compared with captive-held fish in hatchery barren-reared environments with those provided some form of enrichment have been found to have increased brain development and neurogenesis (von Krogh et al., 2010; Näslund et al., 2012; Salvanes et al., 2013), reduced impact from stressors (Braithwaite and Salvanes, 2005; Näslund et al., 2013; Batzina et al., 2014) and improved post-release survival (Rodewald et al., 2011; D'Anna et al., 2012). It has been reported that rainbow trout reared in pond with added structures during the last month preceding smoltification enhanced physiology of smolt comparatively to fish kept in barren raceways (Zydlewski et al., 2003). In an intensive captive rearing environment fish from barren tanks had two fold higher basal levels of plasma cortisol than fish from the enriched rearing environment (Näslund, 2013). Similarly, an enrichment also effect the recovery time in rainbow trout exposed to the stressor e.g. anaesthesia, handling, and other standard stressors. However, cortisol values show less variation between individuals within the enriched groups (Pounder et al., 2016). Besides cortisol, brain monoamines (5-HT, DA and NE) also modified in mice and rates (Naka et al., 2002; Bernes et al., 2008) and in fish (Winberg and Nelson, 1993; Höglunds et al., 2005) by rearing them in enriched environment.

Brain serotonin and catecholamine systems becomes activated when fish confront a stressful condition (Winberg and Nilsson, 1992; Papoutsoglou et al., 2006;

Karakatsouli et al., 2007). Previously, several studies mainly on rats and mice, have reported that housing in enriched environment is associated with increased brain weight, neuronal density and cortical thickness, changes in hippocampal levels of neurotrophins, neurogenesis, serotonin receptors expression and brain monoamine neurotransmitter content (Mohammed et al., 2002; van Praag et al., 2000). Recently reduced brain serotonergic (5HIAA/5HT) system activation as well as reduced DA levels in specific brain areas has been reported (Brenes et al., 2008; McQuaid et al., 2012) in rat and mice kept in enriched environment. It has also been reported that environmental enrichment and structural complexity to the rearing environment induced changes in the brain monoamines levels in gilthead seabream (*Sparus aurata*) (Batzina, 2014). Although, brain monoamines have been shown to be modified in mice and rats reared in enriched environments (Naka et al., 2002; Brenes et al., 2008), but limited literature is available for fish (Höglund et al., 2005).

Realizing the impact of rearing environment on the physiology and life skill activities of different fish species, we assumed that poor post release survivorship of mahseer *Tor putitora* in restocking programs may also be related to the distinct rearing environments at hatcheries that will generate differences in HPI-Axis and many aspects of behavioural skills of mahseer (e.g. anti-predatory, exploratory and foraging behaviour etc.). We hypothesized that by providing hatchery reared mahseer with an enriched environment during early life stages should lower stress response, improve recovery time period from acute stressor and life skill activities (foraging, exploratory, predatory and anti-predatory behaviors). Therefore, we designed study with the aim 1) to evaluate the physiological stress response and behavior of wild caught and hatchery reared mahseer and 2) to modulate the stress response and behavior of fish by manipulation of rearing environment. The physiological stress response of both populations (wild caught and hatchery reared) was evaluated through invasive and non invasive methodology by, examining plasma and water borne cotisol, plasma glucose, monoaminergic activity i.e., 5-HIAA/5-HT, DOPAC/DA, HVA/DA ratios and NE level at different time intervals after exposure to acute stress (5 min chasing and 2 min confinement with hand net). The basal levels and recovery period i.e. return back to basal levels were also studied for defining difference between populations. Furthermore, experiments under laboratory conditions were designed to evaluate the anti-predatory, exploratory and foraging behaviour of both

wild caught and hatchery reared populations. To test the impact of enrichment on physiology and behavior of fish, we devised three different rearing environments (barren, semi-natural and physically enriched) that differ in their levels of complexity and heterogeneity and reared mahseer hatchlings up to advanced fry stage in these three different rearing environments. The physiological stress response and behavior of these three rearing groups were also evaluated by adopting the methodology as used for evaluating the difference between wild caught and captive reared mahseer.

Chapter# 1

Physiological Stress Response of Captive-reared and Wild-caught Mahseer (*Tor putitora*)

ABSTRACT

The altered developmental patterns of hatchery-reared fish suggests that there may be important physiological changes occur that differentiate it from their wild counterpart to cope the life challenges after release into the wild. Differences in the physiological stress response of captive and wild caught individuals have been observed recurrently. Here an attempt has been made to evaluate the physiological stress response of captive-reared and wild-caught endangered fish mahseer (*Tor putitora*) by adopting invasive and non-invasive methods. Before examining the stress response, basal/pre-stress levels of plasma and water-borne cortisol, blood glucose and brain monoamines were noted. Both populations were exposed to short term acute physical stress, i.e. 5 min chasing and 2 min confinement with a hand net and blood, water and brain sample were collected at 0, 0.25, 0.5, 0.75, 2, 4, 6, 8, 24, 48 hrs. The physiological stress response of mahseer showed the activation of hypothalamic–pituitary–interrenal (HPI) axis, serotonergic and dopaminergic systems indicated by several fold elevation of plasma and water-borne cortisol, plasma glucose 5HIAA/5-HT, DOPAC/DA and HVA/DA ratios and NE in both populations after being acute stress (chasing and confinement). Although both populations attained peak levels of all stress parameters except water borne cortisol at the same time period, i.e. at 0.75 hr after stress but showed significant difference ($P<0.05$) in magnitude (concentration) of cortisol, brain 5HIAA/5-HT, DOPAC/DA, HVA/DA ratio and NE. However, water borne cortisol in both populations after being acute stress peak at different time periods, as in wild-caught fish rise to $56.23\pm3.84 ngL^{-1}$, 2 hrs post stress in contrast to captive reared mahseer with peak, $46.48\pm3.32 ngL^{-1}$ at 4 hr after stress. By close examining the results, it appears that wild fish showed typical stress response, i.e. rapid and strong activation of of hypothalamic–pituitary–interrenal (HPI) axis, serotonergic and dopaminergic system and rapid recovery (return back to basal levels) as compared to captive reared counterpart which showed low level of stress hormones and had taken comparatively longer time to attain their basal levels back. These results specify the role of natural rearing environment in shaping the stress response of fish and suggest an improvement in the hatchery rearing environment to reduce the physiological variations and increase the post survivorship of captive reared mahseer.

1. Introduction

Captive animals are the wild animals being kept and bred in captivity. Fish are artificially bred and reared for human consumption, restocking in natural water bodies and for conservation of critically endangered species (Simpson and Jackson, 1996; Lintermans and Ebner, 2006). However, fish from open waters are considered as a "wild animals" (Clarkson, 2003). Wild animals undergo interactive and physiological changes with respect to the environment (Price, 1999; 2002) while life in captivity leads to adaptations according to the artificial environment. Captivity may result in genetically and phenotypically different fish than wild populations because of genetic selection for particular traits or due to the effect of the environment on the phenotype (Berejikian et al., 1999). Therefore, the process of rearing in captivity leads to change the behaviour, physiology and phenotype in comparison with their wild progenitors (Brown and Laland, 2001; Brown and Day, 2002; Zuberi et al., 2011). The reduced genetic vigour, altered immune response and behaviour deficiency appeared as major obstacles in the survival of hatchery reared population in wild (Doyle et al., 2001; Huntingford, 2004; Mathews et al., 2005; Jule et al., 2008).

Domestication and captive rearing of fish for replenishment of natural stock, for conservation of endangered species as well as for aquaculture have increased rapidly worldwide (Boyes, 2016; Jiang, 2010). Domestic animals, including fish are well adapted to artificial environments, arbitrated by planned or inadvertent selective breeding over several generations (Price,1984). In the course of domestication, animals can acquire phenotypic traits differ from their wild ancestors (Price, 1984). Domesticated animals generally show increase body weight, higher growth and reproduction rates compared with their wild counterparts (e.g. birds: Jensen and Andersson, 2005; fishes: Johnsson et al., 1996; Fleming and Einum, 1997; Wright et al., 2006). In birds and mammals, domestication has led to decrease reactivity of the stress axes (Künzl et al., 2003; Ericsson et al., 2014; Fallahsharoudi et al., 2015). Similar changes have also been reported in domestic and wild strains of brown trout, *Salmo trutta* (Lepage et al., 2001), rainbow trout *O. mykiss* (Jentoft et al., 2005), fighting fish *Betta splendens* (Verbeek et al., 2008) and rainbowfish *Melanoteania duboulayi* (Zuberi et al., 2011).

Fish reared under hatchery conditions are subjected to several environmental, social, and husbandry related stimuli that may have potentially noxious or stressful effects. The stress response has been described for numerous fish species (Schreck, 2000; Barton, 2002; Zuberi et al., 2011). Under hatchery condition fish are exposed to acute stress occurs during counting, grading or harvesting while chronic stress, occurs when fish are reared at too high densities, in poor quality water (level DO, high ammonia, high suspended solids, etc.), or when fish are confined, transported, sick or exposed to social interactions between individuals (Berejikian et al., 2000; Salvanes and Braithwaite, 2005; Strand et al., 2010). Chronic stressors are more potent to jeopardize animal health and welfare. Fish under intensive culture conditions are exposed to a regime of acute and chronic stressors, which have adverse effects on growth, reproduction, immune system, flesh quality, feeding behaviour, etc. (Barton and Iwama, 1991; Lowe et al., 1993; Pickering, 1993; Balm, 1997; Pankhurst and Van der Kraak, 1997; Sigholt et al., 1997; Schreck et al., 2001). Fish populations bred in captivity and adapted to the domestic environment by plane or accidental selection could affect the stress responses. Wild fish may respond differently to stressors because of genetic or environmental differences. (Clements and Hicks, 2002).

A wide range of stimuli challenges a fish around the clock, whether in captivity or in the wild (Galhardo and Oliveira, 2009). The physiological and behavioural responses to stress are very well studied in many teleost species, having striking similarities to those of other vertebrates (Barton, 1997; Sumpter, 1997; Barton, 2002; Lee and Berejikian, 2008). It is well known that physiological stress response in fish is biphasic with an initial short time increase in plasma catecholamines from the stores of chromaffin tissue of the kidney, followed by a longer latency i.e., prolonged activation of HPI-Axis and de novo synthesis as well as the release of cortisol (Sumpter, 1997; Wendelaar Bonga, 1997; Pickering, 1998). Although rapid physiological response is mostly arbitrated by cholinergic nerve fibres innervating the chromaffin tissue; but involvement of, adrenocorticotrophic hormone (ACTH), serotonin and non-cholinergic innervation of chromaffin tissue in modulation of catecholamine release is also reported (see review Perry and Bernier, 1999)

The increase in plasma catecholamines levels in initial stress response causes elevation of plasma glucose level because of catecholamine-mediated glycogenolysis (Wendelaar Bonga, 1997) but later on cortisol-mediated gluconeogenesis is responsible for maintaining increase level of glucose. Measurements of plasma cortisol, lactate, and glucose are now in practice to determine the primary and secondary stress response of fish (Clements and Hicks, 2002). Like in other vertebrates, the blood concentration of corticosteroid hormones is a major index of stress in fish, while elevated levels of corticosteroids indicate the activation of the hypothalamus-pituitary-interrenal (HPI) axis (Wendelaar-Bonga, 1997; Höglund et al., 2000; Winberg et al., 1997; Øverli et al., 2000). The main corticosteroid in teleost fish is cortisol (Barton, 2002; Martinez-Porchas et al., 2009; Pankhurst, 2011), and this steroid is used as a causal factor for the determination of the impact of stress (Barton and Iwama, 1991; Harris and Bird, 2000; Pankhurst and Van der Kraak, 2000; Schreck et al., 2001; Consten et al., 2002; Bernier et al., 2004).

Physiological stress and behavioral responses in vertebrate, including fish, are also linked to a large degree by common control mechanisms in the brain while monoamine neurotransmitters: norepinephrine (NE), serotonin (5-hydroxytryptamine, 5-HT), dopamine (DA), play an important role in the co-ordination (Chaouloff, 1993; Winberg and Nilsson, 1993; Gesto et al., 2013; Winberg et al., 2001; Larson et al., 2003; Perreault et al., 2003; Lepage et al., 2005). Brain serotonergic and dopaminergic system play a key role in the regulation of stress reactions (Winberg and Nilsson, 1993; Bowman et al., 2002; Gesto et al., 2013; Winberg et al., 2001; Larson et al., 2003; Perreault et al., 2003; Lepage et al., 2005), mediated by stressor like predator exposure, isolation, handling, pollutant exposure or crowding (Schjolden et al., 2006; Gesto et al., 2008; Gesto et al., 2009; Weber et al., 2012). Number of teleost species as well as mammals showed increase level of brain 5-hydroxyindoleacetic acid (5-HIAA; major 5-HT metabolite) and 5-HIAA/5-HT ratios (Winberg et al.,1997; Øverli et al., 2001; Alanärä et al., 1998; Gesto et al., 2013; Höglund et al., 2002; Lepage et al., 2005; Bowman et al., 2002) in response to stressor like handling and predator exposure. It is previously reported that stressful events affect synthesis of brain serotonin (5-hydroxytryptamine, 5- HT) and turnover

(Barton et al., 2008) as well as rapid activation of brain dopaminergic (DA) and norepinephric systems (Øverli et al., 1999).

Brain sertonergic activity (5-HIAA/ 5-HT ratio) have been found to correlate with plasma cortisol concentration, indicating the involvement of brain 5-HT in the regulation of HPI axis (Winberg et al., 1997; Winberg and Lepage, 1998; Øverli et al., 1999; Höglund et al., 2000; Guesto et al., 2013). It is believed that 5-HT stimulate the hypothalamic–pituitary–adrenal (HPA) axis in mammals (Dinan,1996; Heisler et al., 2007; Herman and Cullinan., 1997) as well as the hypothalamic–pituitary– interrenal axis (HPI axis) in fish (Höglund et al., 2000; Winberg et al., 1997; Øverli et al., 2000). However, the involvement of central DA in the regulation of the HPA axis is still controversial and in mammals central DA has been suggested to act stimulatory, inhibitory or no role in the regulation of the HPA axis (Brambilla et al., 2000; Sullivan and Dufresne, 2006).

Mahseer is one of the most important semi-cold fresh water fish of Pakistan and South Asian countries. It has great importance as a food, research model and in sport fishing (Singh et al., 2009; Barat et al., 2016). However, its natural population has experienced severe declines across their natural range because of the destruction of feeding and spawning grounds, may be due to natural disasters (Rahman et al., 2005) or anthropogenic disturbances, including damming of rivers, deforestation, pollution, and overexploitation (Lakra et al., 2010; Nautiyal, 2014; Pandit and Grumbine, 2012; Khajuria et al., 2013; Gupta et al., 2015; Sharma et al., 2015). Current conservation status of the mahseer is vulnerable and endangered (Ameen et al., 2000; IUCN, 2016). Conservation of endangered and economically important fish is, therefore, a serious challenge. It is important to make serious efforts for the conservation of this remarkable fish species. Many scientists have suggested that special attention is necessary to protect mahseer from extinction (Everard and Kataria, 2011; Naeem et al., 2011; Arora and Julka, 2013; Khajuria et al., 2013, Gupta et al., 2014; Ali et al., 2014; Hussain and Mazid, 2001; Islam, 2002; Yaqoob, 2002; Bhatt and Pandit, 2016).Thus to maintain the natural population of mahseer as well as its conservation and rehabilitation many restocking programs have been initiated but results are still not encouraging and natural population is continually showing declining trend. It may be due to poor post release survival of hatchery reared fish.

We hypothesized that distinct captive rearing environment may generate physiological changes that differentiate hatchery reared mahseer from their wild counterpart to cope the life challenges after release into the wild. The objective of the present study was to evaluate the stress response in term of activation of HPI-axis and brain serotonergic and dopaminergic system of both populations. We examined the physiological stress response of wild caught and captive-reared mahseer population that had been held in captivity for around 4 generations by adopting both invasive and non invasive techniques. We assumed that the captive reared mahseer would show an atypical stress response as compared to the wild population, which should exhibit relatively rapid physiological stress responses followed by a quick recovery.

2. Materials and Methods

2.1. Fish collection from wild environment

Wild mahseer (mean ± SEM body mass and length 15.9 ± 1.37g; 11.2 ± 0.89 cm) was captured through drift gill net (size, 25m L× 4m W; mesh size, 2 cm) from River Haro, Attock 33°46'8" N, 72°14'43" E in DMS (Degrees, Minutes, Seconds) and transported alive (3 g/L) in oxygen filled plastic bags (36 cm L × 24 cm W; 25 L water) to the Fisheries and Aquaculture Research Center, Department of Animal Sciences, Faculty of Biological Sciences, Quaid-I-Azam University Islamabad, Pakistan. Fish were initially housed in replicates of three (with a stocking density of 1.5 g/L: 35 fish/tank) in well aerated circular fibreglass tanks (volume, 500 L water) containing 1cm deep gravel (black and white colour) substrate, river stones (5-6 cm diameter) and artificial plastic plants (25 cm height: n = 10) for at least one week before any further experimentation. Overhead fluorescent tubing was used to provide light (12:12 light, dark) and water temperature was maintained at 22.5 °C. Water quality in terms of total ammonia (< 0.20mg L^{-1}), pH (from 6.8 to 7.48) and dissolved oxygen (>6.4mgL^{-1}) were found to be in the acceptable range for mahseer (Islam, 2002; Bhatt et al., 2004). During acclimation period, wild fish were weaned from live food onto prepared pelleted feed (sinking pellets; Oryza Organics fish feed, size 2 mm; 45 % crude protein, 14 % fats, crude fiber 2 % and 10 % moisture. Fish were fed 4 % body weight twice daily (9:00 am and 5:00 pm).

2.2. Fish collection from captive environment

2.2.1 Conventional breeding and rearing of Mahseer

In Pakistan, artificial propagation of mahseer in captivity was started in 2001 at Mahseer Fish Hatchery Hattian, Attock, Punjab, Pakistan. Generally, at hatcheries, fish brooders are prepared in the structureless concrete rectangular tank (50×50 feet) on live plus prepared pelleted feed. Hand stripping is the method in practice for obtaining eggs and sperms. Incubation of the fertilized egg are carried out in fibreglass hatching tray (size, 1.5 ft W × 15 ft L) in a hatchery room under direct water sprinkling. After hatching (70 to 100 hrs depending upon water temperature),

the hatchlings are shifted to barren circular concrete tank (7 ft diameter × 4 ft H) under slow moving water. After semi-quiescent stage (egg yolk absorption), when fry starts feeding exogenously, they are relocated to semi-earthen ponds (50 W × 100 L ft), earthen bed with concrete walls, having natural food organisms (phytoplankton, zooplankton, diatoms, some protozoa etc.) but without common aquatic weeds or other substrate. They are raised up to fingerling stage on live feed, plus prepared feed by adopting monoculture technique or in combination with other species like common carp (*Cypriuns carpio), rohu (Labeo rohia*) etc.

Breed of the fourth generation, captive mahseer (mean ± SEM body mass and length 15.2 ±0.43g and 9.5 ± 0.63cm) were collected from the hatchery and transported to the Fisheries and Aquaculture Research Center, Department of Animal Sciences, Faculty of Biological Sciences, Quaid-I-Azam University Islamabad, Pakistan. The fish were acclimated for at least 2 weeks in well-aerated circular fibreglass tank (volume, 500 L water) with same stocking as used for wild fish, before starting the experiments. The fish were maintained under a controlled photoperiod (12:12 h day: night) and temperature (22.5 °C). Water quality parameters were maintained at optimum level.

2.3. Experimental protocol for water-borne cortisol collection

2.3.1 Distribution of fish and stress assay procedure

The experiment was designed by adopting the procedure reported by Zuberi et al.(2011) for monitoring the stress response of captive reared and wild rainbow fish (*Melanoteania duboulayi*). Briefly, one week prior to stress assay, both wild (No.40; Average body mass 15.9 ± 1.37g and body length 11.2 ± 0.89 cm) and captive reared mahseer (No.49; Average body mass15.2±0.43g and length 9.5 ± 0.63cm) was re-distributed in 12 similar size glass aquaria (60 cm L ×30 cm W× 30 cm H; 54 L water) at a stocking density of 10.6± 0.03 gL^{-1}. Six glass aquaria were for wild and the other six for captive reared mahseer. The size difference between wild and captive reared fish was controlled by maintaining the stocking density of both populations instead of the number of fish in aquaria. *T. putitora* is an endangered species, difficult to collect and transport live from wild. Therefore, we compromised on size difference.

According to Bender et al. (2008) small variation in body size does not result in considerable changes in metabolism and production of cortisol. The experiment was conducted in replicate, with slight variation in number of fish in aquaria (7, 7, 7, 7, 6 and 6 for wild and 7, 7, 7, 7, 7 and 7 for captive reared mahseer). The six aquaria of each group were split into half for control and other for test stress assay. All aquaria were well equipped with aerator and water heater, to maintain a constant temperature of 22.5˚C and had permanent volume marks for maintaining water volume .

Dechlorinated water to each aquarium was supplied through the main pipeline from water tank set at the same temperature (22.5˚C) used in experimental aquaria. The pipeline at the top of each aquarium had tap with regulator to control the rate of flow of water. Outflow of water were maintained by connecting experimental aquarium via Tygon® tubing and a regulator. During experiment, constant rate of flow, i.e. 25.01± 1.29 ml min^{-1} was maintained. Fish were fed once daily at 09:00 am with floating food (Oryza organics fish feed, pelleted; 55% crude protein, 12% lipid fats, crude fiber 2% and 10% moisture). Every day uneaten food and faces were removed by siphoning and about 20% water in each aquarium was also replaced by the addition of fresh water from the water reserviour. During the experiment, the pH was >7, DO was near saturation (>6.4 mg L^{-1}) ammonia was less than 0.20 ppm. The experiment consists of two treatments; control, i.e. unstressed fish and treated i.e., exposed to acute stress. Treated groups were stressed by following standardized handling stress protocol previously described by Guesto et al. (2013) for rainbow trout with some modification, i.e., 5 min chasing and confining the fish to one corner of the tank with hand net for 2 min.

2.3.2 Water sampling

Twenty four hr prior to the experiment, fish were not fed and on the day of experiment, without disturbing fish, water from each aquarium was exchanged with fresh water from main tank by controlling rate of flow. For obtaining basal level of cortisol, before exposing stress, 500 ml water from each aquarium was collected in glass bottle through long outflow Tygon® tube, without approaching too closely to experimental aquaria. The rate of flow was recalibrated and further water samples were collected by adopting same procedure at 0.5, 0.75, 1, 2, 4, 6, 8, 24 and 48hr

after acute stress (5 min chasing and 2 min confinement). To minimize the possibility of any interference from background, water from the main water reservoir was also collected for cortisol analysis. Throughout the experiment, the flow rate was periodically checked by recording the time to fill the graduated beaker and adjusted the inflow or outflow of water accordingly. Moreover, the volume marks on aquaria also helped in maintaining the constant rate of flow. Immediately after sample collection, water was filtered.

2.3.3 Extraction procedure of Water-borne Cortisol

Zuberi et al. (2011) procedure was used for extraction of water-borne cortisol. Briefly water samples were immediately filtered by using Whatman filter paper No. 1(pore size ,11µm)and passed through a millipore filter (pore size , 0.45 µm) using millipore filter assembly. LiChrolut® RP-18 solid phase extraction cartridges (3 ml, 500 mg, 40 - 63 µm, standard PP Merck) were used for extraction of free cortisol from water samples. Cartridges were primed with 2 × 2 ml HPLC grade methanol (CH_3OH) followed by two consecutive washes with 2 ml double distilled H_2O and fitted to a 20-port vacuum manifold and filtered water samples (500 ml) were pushed through Tygon® tubing into the columns. Once the entire water sample was passed, the cartridges were washed twicewith 4 ml dd water and free cortisol was eluted from the columns with ethyl acetate. The eluted solvent was evaporated at 45°C using a nitrogen gas stream and the residue was re-dissolved in 100ul of ELISA buffer and stored frozen until assayed. The % age efficiency of extraction from water sample was assessed by adding predicted concentrations of 10 and 20 and 50 ng mL^{-1}. standard cortisol to 500 ml water samples collected from main reservoir which supplies water to experimental aquaria. Water samples were then passed through extraction cartridges and cortisol was recovered by using ethyl acetate and the amount was quantified by ELISA. The % recovery was found 90.1%, 95.96% and 96.45% respectively. All values of water-borne cortisol were corrected accordingly.

2.4 Experimental protocols for blood glucose, plasma cortisol and monoamines

2. 4.1 Distribution of fish and stress assay procedure

In order to avoid unnecessary catching of endangered species from wild, the same stock used for water-borne cortisol assay was used for further research work. One week prior to experiment, 60 each wild (mean ± SEM body mass and length 15.9 ± 1.37g; 11.2 ± 0.89 cm respectively) and captive reared (mean ± SEM body mass and length 14.2 ± 0.98g and 9.5 ± 0.63cm respectively) *T. putitora* were re-housed (6 fish per aquarium; stocking density, 10 ± 0.05gL^{-1}) in 20 same size (Length: 120 cm L × 60 cm W× 60 cm H; 96 L water) 430 L volume experimental aquaria (10 aquaria per population, wild : captive reared) well equipped with aerators and heater for constant temperature and almost similar DO level. The fish were kept under flow through system in order to avoid any re-uptake of cortisol and constant rate of flow 100 ml min^{-1} was maintained by connecting each aquaria with Tygon® tube for outflow of water while inflow was controlled through the regulator on tap at the top of each aquarium. After distribution the fish were remained without any disturbance under same laboratory condition (12:12 h, light: dark; temperature, 22.5 °C; DO, > 6.5 mgL^{-1}, total ammonia < 0.25 mgL^{-1}). After acclimatization, in order to obtain the basal level of blood glucose, plasma level of cortisol and brain monoamines, fish in one aquarium of each population were anaesthetized by adding MS-222 (50 mgL^{-1}) by stopping the flow of water . Immediately, blood was drawn from the caudal vein below the lateral line and behind the anal fin by using 1 ml heparinized syringe from every fish of both population and dissected out the brain (within 3 min). The brain was immediately deep frozen in liquid nitrogen and stored at -80°C for further analysis of monoamines, while approximately 20 µl of the blood of every fish was used for blood glucose level and reaming were centrifuged at 3000 rpm for 15 min for the separation of plasma. The plasma samples were collected in a separate tube and cortisol was extracted with ethyl acetate by adopting Backström et al. (2011) protocol. Briefly, 3mL ethyl acetate was added in 300 µL plasma , vortex mixed for 5 min and centrifuged at 1000 rpm for 2 min. The organic layer decanted in a appendourf tube and evaporated under a gentle stream of nitrogen gas at 45°C. The residue was dissolved in buffer provided in the ELISA kit and stored at -20 ºC for analysis of cortisol.

In order to find out the comparison of the stress response of wild and captive reared *T. putitora*, fish in remaining aquaria were exposed to acute handling stress by following the procedure previously adopted by Gesto et al. (2013) for rainbow trout

with some modification. Briefly, fish were chased 5 min with hand net (10cm×10 cm) and confined to one corner of aquaria for 2 min. Fish samples for blood glucose, plasma cortisol and brain monoamines of both populations were collected at 0.5, 0.75, 1, 2, 4, 6, 8, 24 and 48hr after acute stress by adopting the same technique some modification in order to avoid excess use of Ms222. For sampling water level of particular aquarium was lower to 5 L by quick adjustment of inflow and outflow of water and th addition of MS222. Samples (n=6 per population per time period) were collected, by using aquarium assigned for a particular time period (1 aquaria/time period/population).

2.5. Blood Glucose Level

The blood glucose level was analysed with the help of digital glucometer (ACCU-CHEK®Softclix; blood glucose meter). A drop approximately 20 µl of fresh blood was placed on the glucometer strip. The Glucometer showed the result on screen in mg dL^{-1}.The unit of glucose converted from mg dL^{-1} to mmole L^{-1} by dividing the value of glucose by 18.018.

2.6. Water-borne and plasma cortisol analysis

Free cortisol concentration from water and plasma was measured using a commercial ELISA kit (Product # 402710, Neogen Corporation, Lexington, USA, delivered by General Scientific Traders, Pakistan). All samples were run in duplicate. On day of analysis , all reagents and samples were removed from the freezer and kept at room temperature. Reagents were slightly shaken before using and 25µl of cortisol standards and samples were pipetted out and poured in 96 wells of ELISA plate. After that, 200 µl of cortisol enzyme conjugate was loaded into each well and allow them to stand for 10 sec. The solution was incubated at room temperature for 60 min. After incubation, liquid from all wells was removed and ELISA plate was washed first by adding 300 µl of 1X solution in each well and afterword, with washing buffer three times. The remaining water droplets were removed by tabbing the plate on absorbent paper. After remaining water, 100 µ TMB substrate was pippeted in each well and incubated at room temperature for 15 min. The enzyme reaction was terminated by adding 50 µl stop solution 1M-HCl, in each well and subsequently the plate was read

in a plate reader at 450 nm (Microplate Reader; AMPPlots 496, AMEDA Labordianosik). The percentage bound for each standard solution was used to generate a logarithmic curve. This curve was used to calculate the concentrations of plasma cortisol.

The ELISA kit used for analysis of cortisol in the samples was validated by verifying that the slope of the curve obtained by serial dilutions of sample (0, 20, 40, 60 and 80 %) with ELISA buffer matched the curve created with kit standards (slopes, Waterborne cortisol: slope = 0.918, r2 = 0.965, P = 0.95; plasma cortisol: slope = 0.942, r2 = 0.998, P = 0.96 indicating the positive linear relationship. The precision of kit (intra-assay coefficient of variance) was calculated by comparing the results from repeated assays and found 9.8 % for plasma body cortisol and 9.1 for water-borne cortisol. The inter assay (reproducibility) coefficient of variation was calculated by an analysis of three samples in two assays and comparing the results. It was found 9.3 and 12.5 % for water-borne and plasma cortisol respectively.

The efficiency of the cortisol extraction procedure used for water samples (% recovery) was evaluated by adding an equal volume of 10, 20, 60 and 80 ng mL^{-1} standards, supplied with the EIA kit to water samples collected from main tank which supplies dechlorinated water to experimental aquaria. Cortisol from water samples were then extracted with ethyl acetate and measured using EIA as described above by adopting same procedure. The cortisol was eluted from the column using 2× 3 mL volumes of and cortisol level. The minimum observed recovery of the methodology (extraction and ethyl acetate elution) was 94.4%.

The efficiency of cortisol extraction from plasma was evaluated by adding standard cortisol supplied with EIA kit in charcoal stripped plasma at predicting concentrations of, 20, 40 , 60 and 80 ng g^{-1}. Cortisol from stripped plasma was extracted and quantified by adopting the same procedure as described for samples. The % extraction efficiency was found greater than 97.1.

2.6. Monoamines assay procedure

This part of research work was conducted in Department of Neuroscience, Biomedical centrum (BMC) Uppsala University, Uppsala Sweden, under the

supervision of Prof. Svante Winberg and Per-Ove Thörnqvist. The procedure described by Thornqvist et al. (2015) was adopted for the analysis of whole brain levels of monoamines, i.e., serotonin (5-hydroxytryptamine, 5-HT) and its metabolite: 5-hydroxyindoleacetic acid (5-HIAA), Dopamine (DA) and its metabolites: 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanilic acid (HVA) as well as norepinephrine (NE). Briefly, said hormones were extracted by homogenizing the whole brain samples in 4% (w/v) ice-cold perchloric acid containing 10 ng ml^{-1} 3,4-dihydroxybenzylamine (DHBA, the internal standard). Sonifier cell disruptor B-30 (Branson Ultrasonics, Danbury, CT, USA) was used for this purpose. The homogenate was centrifuge at 21,000 × g for 10 min at 4°Cand supernatant was collected for analyzing the amount of monoamines in the samples.

Analysis was done by using high performance liquid chromatography with electrochemical detection (HPLC-EC). For monoamines analysis, a reverse phase column (Reprosil-Pur C18-AQ 3 μm, 100×4 mm column, Dr Maisch HPLC GmbH, Ammerbuch-Entringen, Germany) set at 40°C temperature with ESA 5200 Coulochem II EC detector (ESA, Bedford, MA, USA) having two electrodes at reducing and oxidizing potentials of −40 and +320 mV, and a guarding electrode with a potential of +450 mV was employed. The mobile phase for running samples consist of 1.4 mmol L^{-1} sodium octyl sulphate, 75 mmol L^{-1} sodium phosphate and 10 μmol L^{-1} EDTA in deionized water containing 7% acetonitrile with 3.1 pH , that was adjustedby using phosphoric acid .Standard curve was prepared by running known concentrations of standard solutions and samples were quantified with them.Technique was standardised by using DHBA as the internal standard and recoverywas corrected with the help of HPLC software Clarity™ (DataApex Ltd, Prague, Czech Republic).

2.7. Statistical analysis

The results are expressed as mean ± SEM. All statistical analysis was carried out by using lme4 (Bates et al., 2014) and easyanova (Arnhold, 2013) package of R 3.2.5 (R Development Core Team, 2016). Assumption of normality, homogeneity of variances and additivity of the model were checked by *Shapiro-Wilks, Levene's*and *Tukey1-dF Test* respectively. The effects of rearing environment on blood glucose,

whole body cortisol and brain monoamines were analysed by using ANOVA with double factorial in a complete randomized design followed by post hoc *Tukey's HSD.* Values of $P<0.05$ were considered statistically significant

3. Results

3.1. Water-born cortisol

Cortisol was not detected in water supplying to experimental aquaria. Cortisol concentration was significantly higher in wild mahseer than captive-reared counterpart and significantly higher in fish confronted acute stress than control group ($F_{1,\ 220}$ = 1509.159, p<0.001, Table 1, Fig. 1). The significant interaction between Population and Treatment ($F_{1,\ 220}$ = 4.125.159, p<0.05, Table 1) indicated the way, the response of the two populations to acute stress varied. By examining the result more closely it appears that after an acute stress, water-borne cortisol was higher in wild fish than captive-reared mahseer. The basal level of water-borne cortisol of (4.33±0.35 and 7.55±0.36μgL^{-1}), but control groups of both populations did not show significant fluctuation during the experimental period. The repeated measure analysis indicated that cortisol concentration changed over time ($F_{11,\ 220}$ = 109.828, p<0.001), while significant interaction between Time and Treatment ($F_{11,\ 220}$ = 109.828, p<0.001), showed the combined effect of both variables. Moreover, the significant three-way interaction between treatment, time and populations is indicative of the different patterns displayed by fish from both populations in both treatments over time. During the control treatment there were no differences between the two populations (P>0.05), no change in water-borne cortisol over time (P > 0.05). In contrast, after acute there was a significant difference between captive reared and wild populations ($F_{1,\ 220}$=26.891, P<0.001), a change in water-born cortisol over time ($F_{11,\ 220}$ =109.828, P=0.001) and an interaction between these two variables ($F_{11,\ 220}$ =107.44, P<0.001). Water-born cortisol in wild fish was significantly elevated at 0.25 h and peaked at 2 h post stress and then started declining whereas cortisol concentration in captive reared fish gradually built up, although peaked at 4 h post stress, but concentration at 2 hrs was slightly less but statistically compared to observe at 4 hrs (Table 2, Fig. 1). In wild mahseer, water-born cortisol return to basal level after 8hrs post stress as compared to captive reared remained high even after 24 hrs

3. 2. Blood plasma cortisol

The pre-stress blood plasma cortisol level was significantly different in two populations of mahseer wild and captive (Tukey's post hoc: p=0.05; Table 3). The significant interaction between population and treatment (two way ANOVA: $F_{2, 100}$= 3.6288, p<0.01) indicated how the stress response of fish from rearing environment varied. The plasma cortisol concentration changed after exposure to stress as compared to pre-stress (two way ANOVA: $F_{1, 100}$ = 469.1572, p<0.001; Table 3, Fig. 2), also changed over time and showed variable concentration of most of the studied hours among wild and captive populations of mahseer (two way ANOVA: $F_{8, 100}$ = 345.1709, p<0.001). The significant interaction of populations with treatment (two way ANOVA: $F_{1, 100}$ =469.1572, p<0.001) as well as with time (two way ANOVA: $F_{8, 150}$ = 345.1709, p<0.001)indicated how different rearing groups differ in their stress response. Although both populations attained peak levels after 0.75 min of stress, but cortisol concentration and the trend to attain peak values were significantly different in two populations. At 0.25 min wild population fish showed rapidly increased and highest level of cortisol, but afterword trend changed and at the 0.75 min highest level was observed in wild fish followed by captive reared fish (27.65 ± 3.01 and 17.80 ± 2.20 respectively). After word plasma cortisol level showed decreasing trend, but wild-caught mahseer showed rapid recovery and at 6 hrs attained levels statistically comparable to their pre-stress levels as compared to captive fish, where it remained high even after 24 hrs. At 48 hr post stress, plasma cortisol concentrations of all groups were statistically compared to their respective pre-stress values (Table 4, Fig.2)

3. 3.Blood glucose

Before stress, blood glucose level of captive reared mahseer was not significantly different than wild-caught mahseer (Tukey's post hoc: p=0.28). After treatment, i.e., exposure to acute stress, a significant difference between two populations(two way ANOVA: $F_{1, 100}$= 6.03, p<0.01 Table 5, Fig.3), changed in glucose level over time (two way ANOVA: $F_{8, 100}$ = 265.8381, p<0.001) and interactions between different rearing groups of fish with time (two way ANOVA: $F_{8, 100}$ = 18.0537, p<0.001) and treatment (two way ANOVA: $F_{1, 100}$ = 2.1872, p<0.02 Table 5, Fig.3) were observed. Like plasma cortisol, plasma glucose of all rearing groups also differs in peak concentration, highest in wild followed by captive reared

fish. Although, both populations attained peak value at 2 hr, but wild mahseer showed initially rapid increased at 0.25 min as compared to steady increased in concentration, observed in captive reared mahseer. After stress, wild mahseer recovered their pre-stress level earlier, i.e., at 8 hrs than captive reared fish.

3.4. Brain monoamines

3.4.1. Serotonergic activity (5HIAA/5HT ratio)

The pre stress brain serotonergic activity (5HIAA/5HT ratio) of advanced fry of mahseer from two different populations of mahseer wild and captive was considerably similar (Turkey's post hoc: $p = 0.62$, Table 8, Fig 4). Exposure to stress showed changes in 5HIAA/5HT brain ratio (n = 6, mean ± SEM: ANOVA: $F_{1, 100} = 142.4145$, $p < 0.001$, Table 7) over time (n = 6, mean ± SEM: ANOVA: $F_{8, 100} = 222.0895$, $p < 0.001$) at different level in two populations of mahseer (n = 6, mean ± SE: ANOVA: $F_{1, 100} = 10.1893$, $p < 0.001$). The significant interaction between time and population (ANOVA: $F_{8, 100} = 9.7529$, $p < 0.001$) suggesting that in different rearing group, 5HIAA/5HT ratio changed with time (ANOVA: $F_{8, 100}=222.0895$, $p = 0.001$), Treatment x population (ANOVA: $F_{2, 100} = 4.1220$, $p = 0.032$). Although both populations after stress attained their peak level at 0.75 min after stress (Table 8, Fig 4). However, captive-reared fish had significantly low 5HIAA/5HT ratio compared with wild reared fish (Tukey's post hoc: $p = 0.001$). After peak ratio, there was a steady and continuous decline in following time period. Moreover, if we look more closely, it appears that different population showed a somewhat difference in the recovery period (Table 8, Fig 4). Wild populations of fish attained a comparable basal level ratio of 5HIAA/5HT earlier, i.e., at 4 hrs after stress as compared to captive reared fish which attained the level at 6 hrs. It was also observed that at 0.25, 0.5, 2 and 4 hr a clear difference in the ratio of 5HIAA/5HT was noted between the wild and captive population (Tukey's post hoc: $p<0.01$) while at 6, 8, 24 and 48 hr captive and wild did not show significant differences (Tukey's post hoc: $p>0.05$).

3.4.2. Dopaminergic activity (DOPAC/DA ratio)

Before stress, whole brain dopaminergic activity index (DOPAC/DA ratio) in two populations of mahseer were almost similar in range (Tukey's post hoc: $p > 0.05$, Fig. 5, Table 10), while after acute physical stress, it showed changes (ANOVA: $F_{1, 100} = 65.3270$, $p < 0.001$, Table 9) with time (ANOVA: $F_{8, 100} = 95.2416$, $p < 0.001$, Table 9) in all rearing groups and attained peak level at 0.75 hr. The significant interaction between time and population (ANOVA: $F_{8, 100} = 4.0743$, $p > 0.48$) while non-significant interaction between treatment and population (ANOVA: $F_{1, 100} = 0.4881$, $p = 0.4863$, Table 9) indicated that although DOPAC/ DA ratio changed in both populations over time after treatment. Captive and wild fish showed significant difference at 0.25, 0.75, 2,4 hr (Table 10, Fig. 5). However, both populations have no significant difference at 6,8, 24 and 48 hr after stress. Wild reared fish recovered their pre-stress level earlier, i.e. at 4 hrs as compared to captive reared fish which achieved this level at 6 hrs

3.4.3.HVA/DA ratio

Pre-stress brain HVA/DA ratios of mahseer in two different rearing populations were statistically similar (Tukey's post hoc: $p>0.05$, Table 12, Fig. 6). After exposure to acute stress, there was significant changed in ratio (two way ANOVA: $F_{1, 100} = 16.3268$, $p < 0.001$, Table 11), at different time period (two way ANOVA: $F_{8, 100} = 28.77$, $p < 0.001$). There was no interaction between population and treatment (two way ANOVA: $F_{1, 100} = 0.0133$, $p=0.908$) as well as between rearing groups and time (two way ANOVA: $F_{8, 100} = 0.8952$, $p= 0.523$). The captive and wild fish show significant difference at 0.25 and 2hr in HVA/DA ratios (Tukey's post hoc: $p = 0.05$, $p = 0.04$, respectively). Both semi-natural and physically enriched environment groups recovered their pre stress HVA/DA ratio earlier, i.e., at 2 hrs as compared barren reared group which attained this at 4 hr after stress (Table12, Fig. 6).

3.4.4 Norepinephrine (NE)

No significant difference was observed in pre-stress brain norepinephrine (NE) level of wild and captive population (Tukey's post hoc; $p = 0.55$, table 14, Fig. 7). After exposure to acute stress, both populations showed significant increase in NE

level as compared to their pre-stress level (ANOVA: $F_{1,\ 100} = 283.4336$, $p < 0.001$; Table 13, Fig. 7), that change over time, peak at 0.75 hr and then decline gradually up to 48 hrs (ANOVA: $F_{8,\ 100} = 379.8585$, $p < 0.001$). The significant interaction between variables indicates how the different rearing groups differ in physiological stress response, i.e., at most of time period showed significant difference in NE level. If we look more closely at the results, it appears that during recovery, wild mahseer attained comparable pre-stress level of NE earlier than captive mahseer (Table 14, Fig. 7). All brain monoamines showed positive correlation with plasma cortisol (Figures 8 and 9, Table 15 and 16).

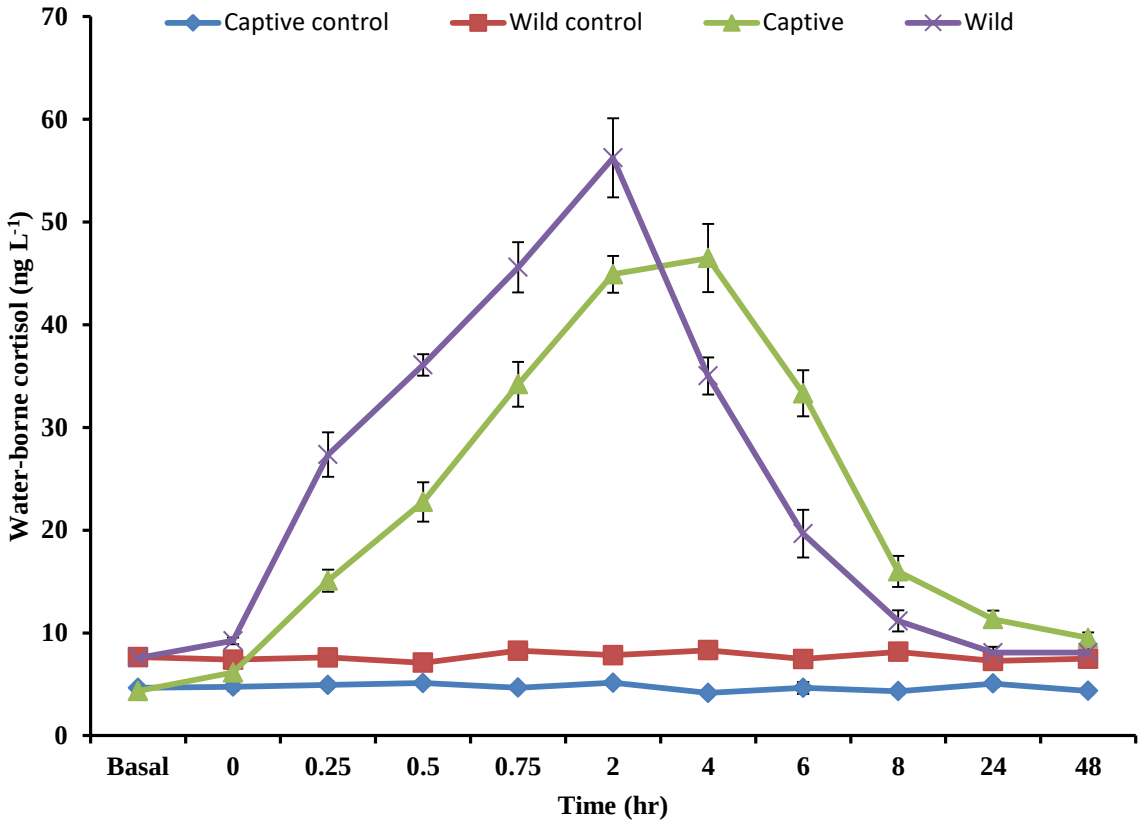


Fig.1. Mean (± SEM) water-borne cortisol (ngL^{-1}) of captive-reared and wild-caught *T. putitora* (n=6) subjected to acute physical stress and sampled at various time intervals. Basal mean pre-stress level.

Table 1. Summary of the ANOVA examining water-borne cortisol in captive-reared and wild-caught *T. putitora* during control and acute physical stress treatments.

Source of variation	**df**	**SS**	**MS**	**F**	**P**
Population	1	330	330	26.891	<0.001
Treatment	1	18546	18546	1509.159	< 0.001
Population: Treatment	1	64.95	64.95	5.4125	0.046
Time	11	14846	1350	109.828	< 0.001
Population: Time	9	1308	145	11.829	<0.001
Treatment: Time	11	14523	1320	107.44	< 0.001
Population:Treatment:Time	9	1470	163	13.293	< 0.001
Residuals	220	2704	12		

Table 2. Water-borne cortisol (mean ng L^{-1} ± SEM) in captive-reared and wild-caught *T. putitora* subjected to acute physical stress and sampled at various time intervals. Basal mean pre-stress level.

Time (hr)	Populations				Statistical Comparisons			
	Control		Treated		Between control groups	Between treated groups	Control vs Treated	
	Cap	Wd	Cap	Wd	Cap-Wd	Cap-Wd	Cap-Cap	Wd-Wd
Basal	4.65±0.33^{a}	7.63±0.40^{a}	4.33±0.35^{g}	7.55±0.36^{f}	0.059	0.11	0.87	0.96
0	4.75±0.36^{a}	8.39±0.49^{a}	6.15±0.31f^{g}	9.22±0.32^{f}	0.07	0.06	0.71	0.68
0.25	4.93±0.35^{a}	7.61±0.51^{a}	15.08±1.06^{de}	27.34±2.16^{d}	0.18	0.001	0.001	0.001
0.5	5.11±0.29^{a}	7.09±0.38^{a}	22.75±1.92^{d}	36.08±1.04^{c}	0.32	0.001	0.001	0.001
0.75	4.66±0.31^{a}	8.25±0.68^{a}	34.2±2.17^{bc}	45.58±2.45^{b}	0.07	0.001	0.001	0.001
2	5.14±0.18^{a}	7.82±0.59^{a}	44.91±1.79^{ab}	56.23±3.84^{a}	0.18	0.001	0.001	0.001
4	4.15±0.36^{a}	8.31±0.40^{a}	46.48±3.32^{a}	35.01±1.81^{c}	0.04	0.001	0.001	0.001
6	4.65±0.57^{a}	7.45±0.27^{a}	33.31±2.24^{c}	19.65±2.32^{e}	0.16	0.001	0.001	0.001
8	4.31±0.34^{a}	8.14±0.39^{a}	15.98±1.49^{d}	11.16±1.01^{f}	0.060	0.001	0.001	0.13
24	5.06±0.35^{a}	7.27±0.42^{a}	11.33±0.83^{ef}	8.08±0.56^{f}	0.27	0.1	0.002	0.68
48	4.34±0.30^{a}	7.49±0.23^{a}	9.51±0.52^{eg}	8.08±0.55^{f}	0.12	0.47	0.01	0.77

P values in the rows from ANOVA with double factorial, complete randomized designed followed by Tukey's post hoc shows a pairwise comparison of water-borne cortisol level of wild and captive-reared *T. putitora*. Means with different superscript are significantly different ($P<0.05$) in the columns compared water-borne cortisol (ngL^{-1}) after the stress with their respective basal level (pre-stress) of each population. Cap= captive reared fish and Wd = wild caught fish.

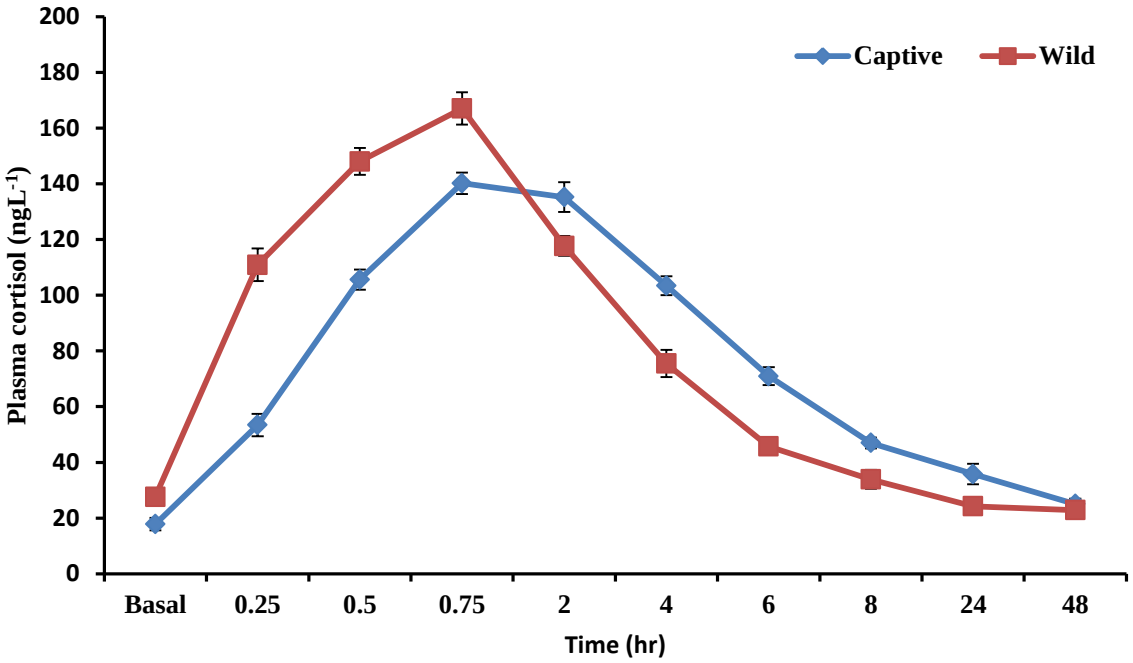


Fig. 2. Mean (± SEM) plasma cortisol (ngL^{-1}) of captive-reared and wild-caught *T. putitora* (n=6) subjected to acute physical stress and sampled at various time intervals. Basal mean pre-stress level.

Table 3. Summary of the ANOVA comparing plasma cortisol in captive-reared and wild-caught *T. putitora* during control and acute physical stress treatments.

Source of variation	**df**	**SS**	**MS**	**F**	**p**
Population	1	502	502	6.592	0.01
Treatment	1	35753	35753	469.157	< 0.001
Population:Treatment	1	228	228	3.628	0. 04
Time	8	210436	26304	345.171	< 0.001
Population: Time	8	23294	2912	38.208	< 0.001
Residuals	100	7621	76		

Table 4. Mean (± SEM) plasma cortisol (ngL^{-1}) in wild and captive reared *T. putitora* subjected to acute physical stress and sampled at various time intervals. Basal mean pre-stress level.

Time (hr)	Populations		Statistics
	Captive	**Wild**	**(p-value)**
Basal	17.80 ± 2.20^{g}	27.65 ± 3.01^{ef}	0.02
0.25	53.42 ± 4.01^{d}	110.91 ± 8.84^{c}	0.001
0.5	105.62 ± 3.58^{b}	148.01 ± 6.82^{b}	0.001
0.75	140.23 ± 5.84^{a}	167.04 ± 5.79^{a}	0.001
2	135.23 ± 5.36^{a}	117.70 ± 3.55^{c}	0.001
4	103.42 ± 6.42^{b}	75.48 ± 4.91^{d}	0.001
6	70.95 ± 5.22^{c}	45.73 ± 4.58^{e}	0.001
8	$46.98\pm3.94d^{e}$	33.88 ± 3.36^{ef}	0.01
24	35.77 ± 3.68^{ef}	24.26 ± 1.41^{f}	0.02
48	25.01 ± 1.97^{fg}	22.87 ± 2.08^{f}	0.67

P values in the rows from ANOVA with double factorial, complete randomized design followed by Tukey's post hoc shows a pairwise comparison of plasma cortisol level (ngL^{-1}) of *T. putitora* from two different populations (wild and captive). Means with different superscript are significantly different ($P<0.05$) in the columns compared plasma cortisol after stress with a basal (pre-stress) level in each population.

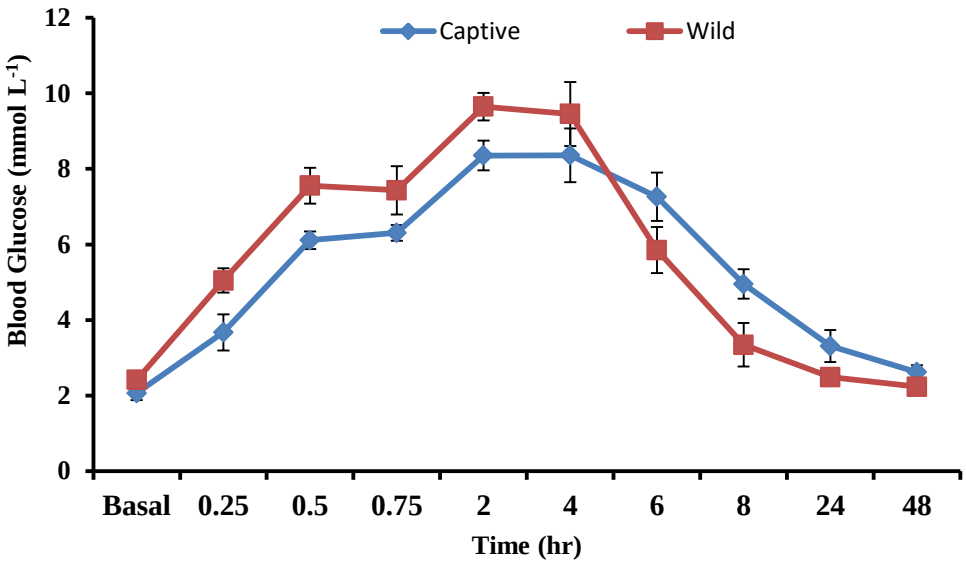


Fig. 3. Mean (± SEM) blood glucose (mmol L^{-1}) of captive reared and wild caught *T. putitora* (n = 6) subjected to acute physical stress and sampled at various time intervals. Basal mean pre-stress level.

Table 5. Summary of the ANOVA examining blood glucose in captive-reared and wild-caught *T. putitora* during control and acute physical stress treatments.

Source of variation	**df**	**SS**	**MS**	**F**	**p**
Population	1	1.590	1.590	6.036	0.006
Treatment	1	132.32	132.318	502.264	< 0.001
Population:Treatment	1	0.575	0.575	2.187	0.026
Time	8	560.27	70.033	265.838	< 0.001
Population: Time	8	38.05	4.756	18.054	< 0.001
Residuals	100	26.34	0.263		

Table 6. Mean (± SEM) of the total blood glucose (mmol L^{-1}) in wild and captive reared *T. putitora* subjected to acute physical stress and sampled at various time intervals. Basal mean pre-stress level.

Time (hr)	Population		Statistics
	Captive	**Wild**	**P-value**
Basal	2.06 ± 0.18^{g}	2.41 ± 0.14^{de}	0.23
0.25	3.93 ± 0.47^{e}	5.04 ± 0.32^{c}	0.001
0.5	6.43 ± 0.23^{cd}	7.55 ± 0.47^{b}	0.001
0.75	6.35 ± 0.20^{bc}	7.43 ± 0.63^{b}	0.001
2	8.35 ± 0.394^{a}	9.64 ± 0.36^{a}	0.001
4	8.36 ± 0.71^{a}	9.45 ± 0.84^{a}	0.001
6	7.25 ± 0.63^{b}	5.85 ± 0.60^{c}	0.001
8	4.95 ± 0.38^{d}	3.34 ± 0.57^{d}	0.001
24	3.31 ± 0.42^{ef}	2.48 ± 0.02^{de}	0.006
48	2.61 ± 0.18^{fg}	2.23 ± 0.15^{e}	0.19

P values in the rows from ANOVA with double factorial, complete randomized design followed by Tukey's post hoc shows a pairwise comparison of blood glucose level of *T. putitora* from the two different populations (wild and captive). Means with different superscript are significantly ($P < 0.05$) different in the columns compared blood glucose after stress with a basal level (pre-stress) in each population.

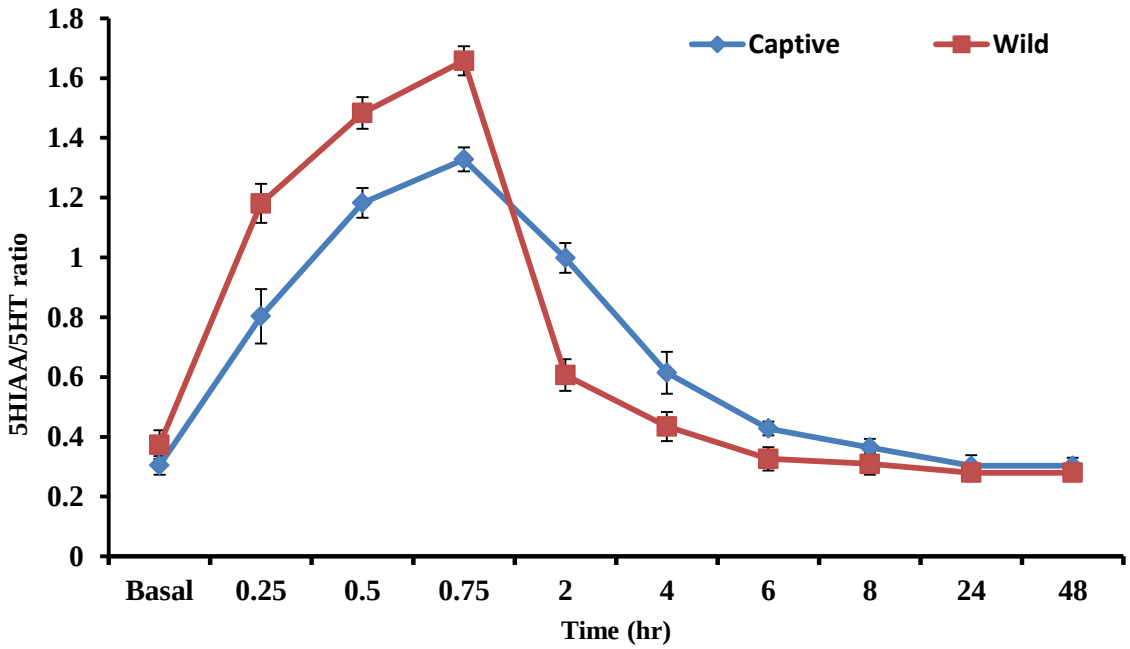


Fig. 4. Mean (± SEM) brain serotonergic 5HIAA/5-HT ratio in captive reared and wild caught *T. putitora* (n=6) subjected to acute physical stress and sampled at various time intervals. Basal mean pre stress level.

Table 7. Summary of the ANOVA representing brain serotonergic activity (5HIAA/5-HT ratio) in captive-reared and wild-caught *T. putitora* during control and acute physical stress treatments.

Source of variation	**df**	**SS**	**MS**	**F**	**P**
Population	1	0.022	0.022	10.189	< 0.001
Treatment	1	1.688	1.688	142.414	< 0.001
Population:Treatment	1	0.048	0.048	4.122	0.033
Time	8	21.062	2.632	222.089	< 0.001
Population: Time	8	0.925	0.116	9.752	0.001
Residuals	100	1.186	0.012		

Table 8. Mean (± SEM) brain 5HIAA/5-HT in wild and captive reared *T. putitora* subjected to acute physical stress and sampled at various time intervals. Basal mean pre-stress level.

Time (hr)	**Populations**		**Statistics**
	Captive	**Wild**	**P-value**
Basal	0.30 ± 0.031^{e}	0.37 ± 0.04^{d}	0.62
0.25	0.80 ± 0.09^{bc}	1.18 ± 0.06^{b}	0.001
0.5	1.18 ± 0.04^{a}	1.48 ± 0.05^{a}	0.001
0.75	1.32 ± 0.05^{a}	1.65 ± 0.04^{a}	0.001
2	0.99 ± 0.06^{b}	0.60 ± 0.05^{c}	0.001
4	0.61 ± 0.07^{cd}	0.43 ± 0.04^{cd}	0.01
6	0.42 ± 0.02^{de}	0.32 ± 0.03^{d}	0.09
8	0.36 ± 0.02^{e}	0.30 ± 0.03^{d}	0.33
24	0.30 ± 0.03^{e}	0.27 ± 0.02^{d}	0.69
48	0.30 ± 0.02^{e}	0.27 ± 0.02^{d}	0.69

P values in the rows from ANOVA with double factorial, the complete randomized design followed by Tukey's post hoc shows a pairwise comparison of the brain 5HIAA/5-HT ratio of *T. putitora* from two different populations (wild and captive). Means with different superscript are significantly different ($P < 0.05$) in the columns compared the brain 5HIAA/5-HT ratio after stress with basal levels in each population.

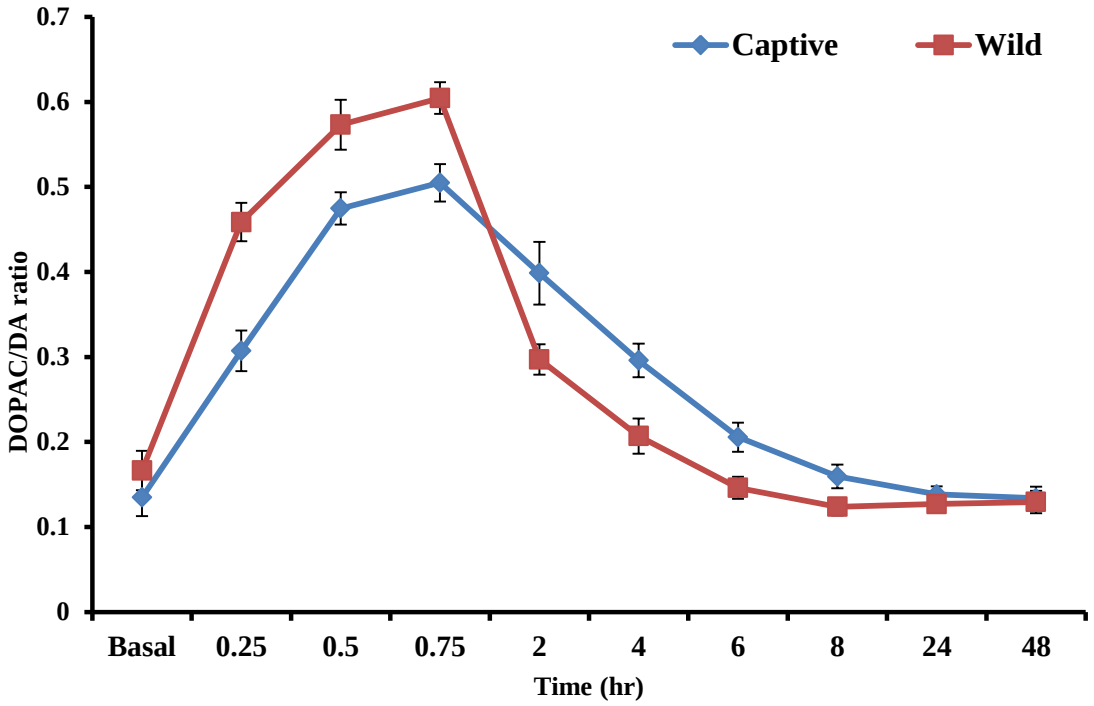


Fig. 5. Mean (± SEM) brain DOPAC/DA ratio in wild caught and captive reared *T. putitora* (n=6) subjected to acute physical stress and sampled at various time intervals. Basal mean pre stress level.

Table 9. Summary of the ANOVA examining brain dopaminergic activity (DOPAC/DA ratio) in captive-reared and wild-caught *T. putitora* during control and acute physical stress treatments.

Source of variation	**df**	**SS**	**MS**	**F**	**P**
Population	1	0.007	0.007	2.021	0.046
Treatment	1	0.242	0.242	65.327	<0.001
Population:Treatment	1	0.002	0.002	0.488	0.48
Time	8	2.818	0.352	95.242	<0.001
Population: Time	8	0.120	0.015	4.074	0.001
Residuals	100	0.369	0.004		

Table 10. Mean value (± SEM) brain DOPAC/DA ratio in wild and captive reared *T. putitora* subjected to acute physical stress and sampled at various time intervals. Basal mean pre-stress level.

Time (hr)	Populations		Statistics
	Captive	**Wild**	**P-value**
Basal	0.13 ± 0.02^{d}	0.16 ± 0.02^{d}	0.53
0.25	0.30 ± 0.03^{b}	0.45 ± 0.02^{b}	0.004
0.5	0.47 ± 0.05^{a}	0.57 ± 0.03^{a}	0.02
0.75	0.50 ± 0.02^{a}	0.60 ± 0.01^{a}	0.05
2	0.39 ± 0.03^{b}	0.29 ± 0.01^{c}	0.02
4	0.29 ± 0.02^{bc}	0.20 ± 0.02^{cd}	0.01
6	0.20 ± 0.01^{cd}	0.14 ± 0.01^{d}	0.09
8	0.15 ± 0.01^{d}	0.12 ± 0.01^{d}	0.311
24	0.13 ± 0.009^{d}	0.12 ± 0.006^{d}	0.75
48	0.13 ± 0.01^{d}	0.12 ± 0.01^{d}	0.90

P values in the rows from ANOVA with double factorial, complete randomized design followed by Tukey's post hoc shows a pairwise comparison of brain DOPAC/DA ratio (mean± SEM) of *T. putitora* from two different populations (wild and captive). Means with different superscript are significantly different ($P < 0.05$) in the columns compared brain DOPAC/DA ratio after stress with a basal level (pre-stress) in each population.

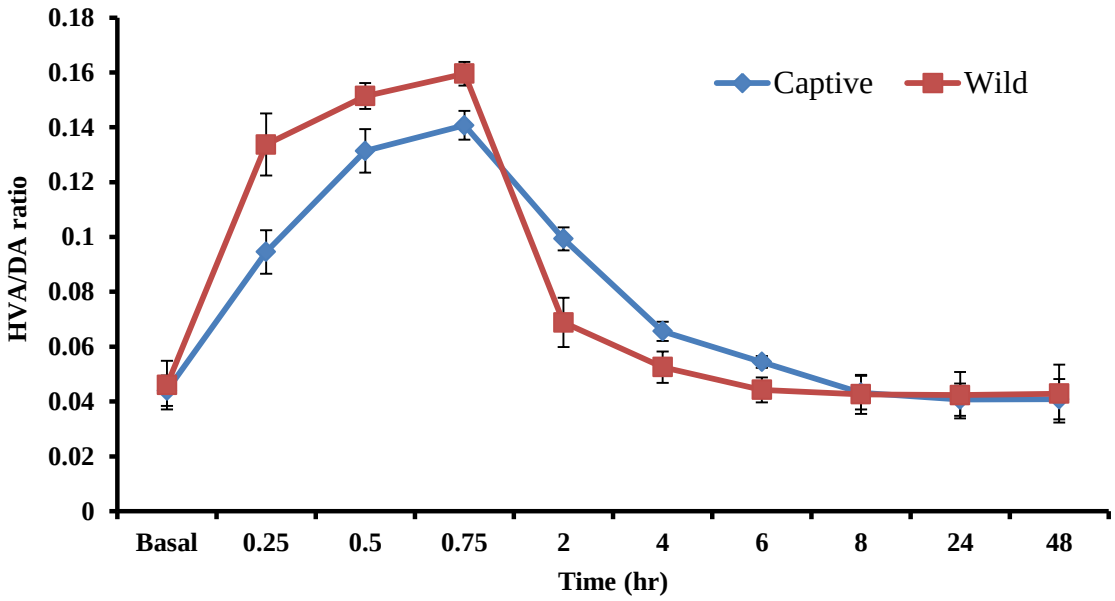


Fig 6. Mean (± SEM) brain HVA/DA ratio in wild caught and captive reared *T. putitora* (n=6) subjected to acute physical stress and sampled at various time intervals. Basal mean pre stress level.

Table 11. Summary of the ANOVA examining brain HVA/DA in captive-reared and wild-caught *T. putitora* during control and acute physical stress treatments.

Source of variation	**df**	**SS**	**MS**	**F**	**p**
Population	1	0.003	0.003	3.030	0.032
Treatment	1	0.014	0.014	16.326	<0.001
Population:Treatment	1	0.000	<0.001	0.013	0.90
Time	8	0.204	0.026	28.779	< 0.001
Population: Time	8	0.006	0.001	0.895	0.52
Residuals	100	0.089	0.001		

Table 12. Mean (± SEM) brain HVA/DA ratio in wild and captive reared *T. putitora* subjected to acute physical stress and sampled at various time intervals. Basal mean pre-stress level.

Time (hr)	**Populations**		**Statistics**
	Captive	**Wild**	**P-value**
Basal	0.0439 ± 0.005^{cd}	0.0460 ± 0.008^{b}	0.87
0.25	0.094 ± 0.007^{ab}	0.133 ± 0.011^{a}	0.05
0.5	0.141 ± 0.007^{a}	0.151 ± 0.004^{a}	0.56
0.75	0.147 ± 0.005^{a}	0.159 ± 0.004^{a}	0.57
2	0.099 ± 0.004^{ab}	0.068 ± 0.008^{b}	0.05
4	0.065 ± 0.003^{bcd}	0.052 ± 0.005^{b}	0.14
6	0.054 ± 0.002^{bcd}	0.044 ± 0.004^{b}	0.32
8	0.043 ± 0.006^{cd}	0.042 ± 0.007^{b}	0.97
24	0.040 ± 0.005^{c}	0.042 ± 0.008^{b}	0.88
48	0.040 ± 0.007^{c}	0.042 ± 0.01^{b}	0.90

P values in the rows from ANOVA with double factorial, complete randomized design followed by Tukey's post hoc shows a pairwise comparison of brain HVA/DA ratio of *T. putitora* from the two different populations (wild and captive). Means with different superscript are significantly different ($P < 0.05$) in the columns compared brain HVA/DA ratio after stress with a basal (pre-stress) level in each population.

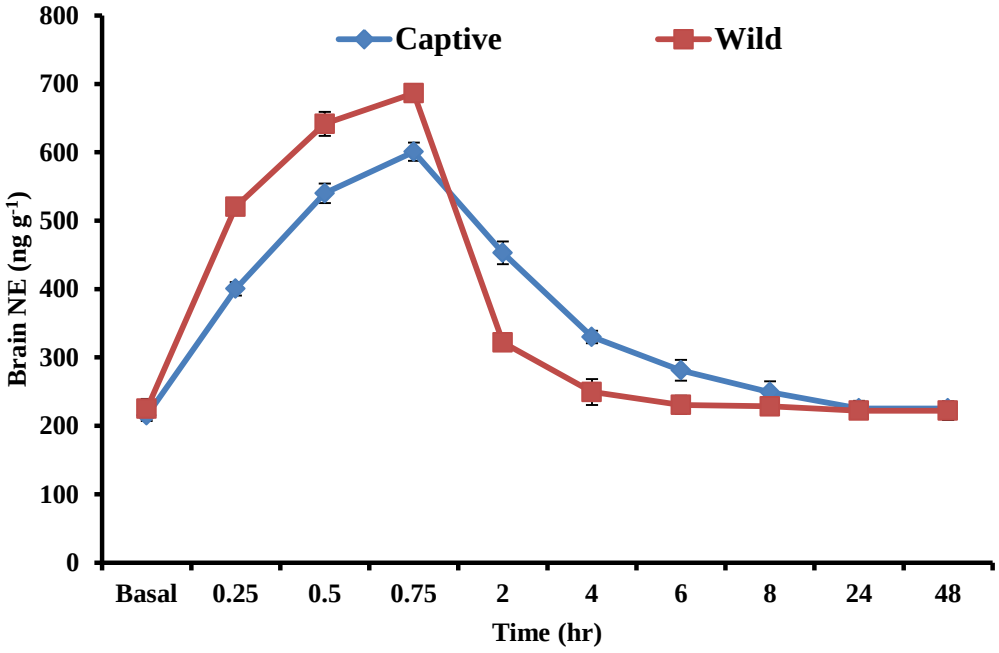


Fig 7. Mean (± SEM) brain NE (ngg^{-1}) in wild caught and captive reared *T. putitora* (n=6) subjected to acute physical stress and sampled at various time intervals. Basal mean pre stress level.

Table 13. Summary of the ANOVA examining brain NE in captive-reared and wild-caught *T. putitora* during control and acute physical stress treatments.

Source of variation	**df**	**SS**	**MS**	**F**	**P**
Population	1	12645	12645	15.255	<0.001
Treatment	1	238842	238842	283.434	<0.001
Population:Treatment	1	4389	4389	5.207	0.001
Time	8	2560773	320097	379.858	<0.001
Population:Time	8	174959	21870	25.953	<0.001
Residuals	100	84267	843		

Table 14. Mean (± SEM) brain NE (ngg^{-1}) in wild and captive reared *T. putitora* subjected to acute physical stress and sampled at various time intervals. Basal mean pre-stress level.

Time (hr)	**Populations**		**Statistics**
	Captive	**Wild**	**P-value**
Basal	215.09 ± 8.20^{f}	225.01 ± 14.22^{d}	0.55
0.25	400.45 ± 9.80^{c}	520.44 ± 10.21^{b}	0.001
0.5	540.07 ± 14.40^{b}	641.53 ± 17.58^{a}	0.001
0.75	600.76 ± 13.49^{a}	686.37 ± 10.20^{a}	0.001
2	452.89 ± 16.73^{c}	321.88 ± 12.00^{c}	0.001
4	329.98 ± 9.25^{d}	249.47 ± 15.09^{d}	0.001
6	281.10 ± 15.25^{de}	230.59 ± 13.35^{d}	0.01
8	249.27 ± 15.6^{ef}	228.43 ± 8.33^{d}	0.19
24	225.32 ± 11.02^{f}	222.11 ± 10.31^{d}	0.84
48	225.49 ± 10.30^{f}	222.11 ± 13.71^{d}	0.80

P values in the rows from ANOVA with double factorial, complete randomized design followed by Tukey's post hoc shows a pairwise comparison of brain NE (ngg^{-1} tissue) *T. putitora* from the two different populations (wild and captive). Means with different superscript are significantly different ($P < 0.05$) in the columns compared brain NE (ngg^{-1} tissue) after stress with a Basal (pre-stress) level in each population.

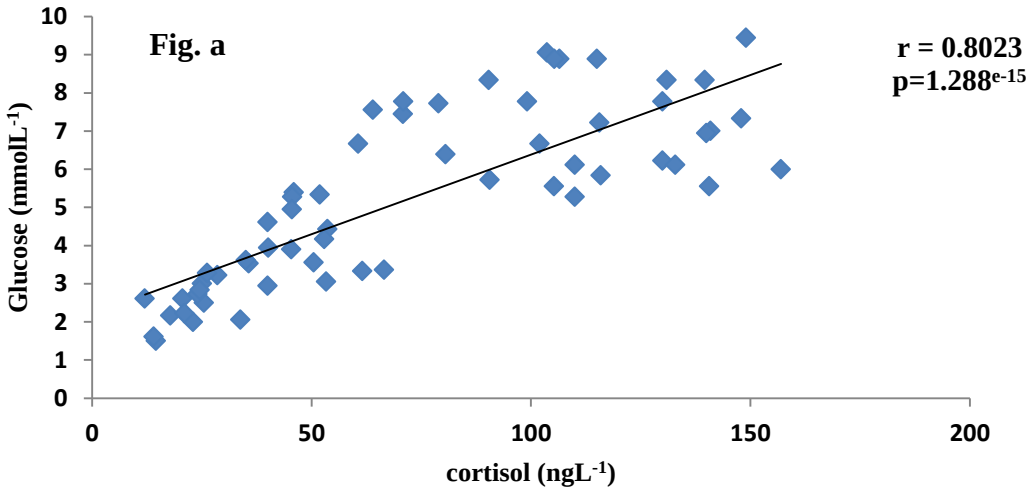


Fig. a
r = 0.8023
p=1.288e-15
Glucose (mmolL-1)
cortisol (ngL-1)
0
1
2
3
4
5
6
7
8
9
10
0
50
100
150
200

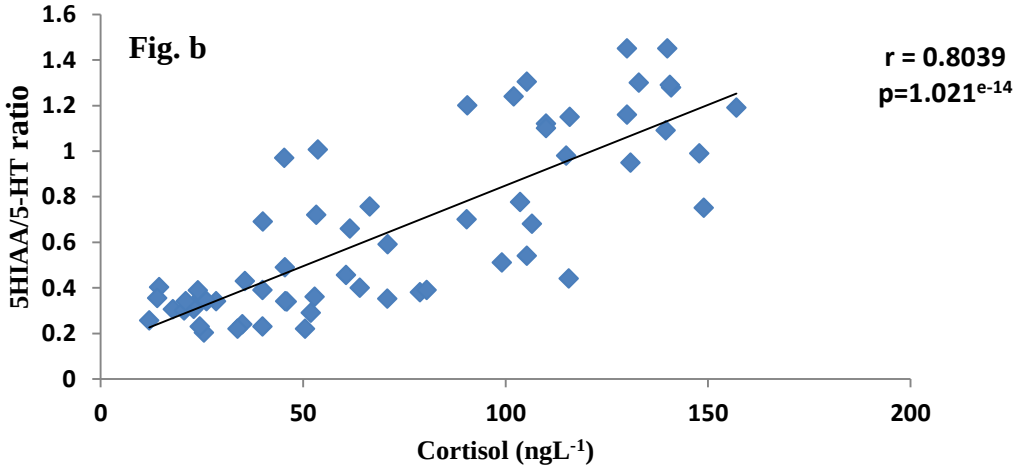


Fig. b
r = 0.8039
p=1.021e-14
5HIAA/5-HT ratio
Cortisol (ngL-1)
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
0
50
100
150
200

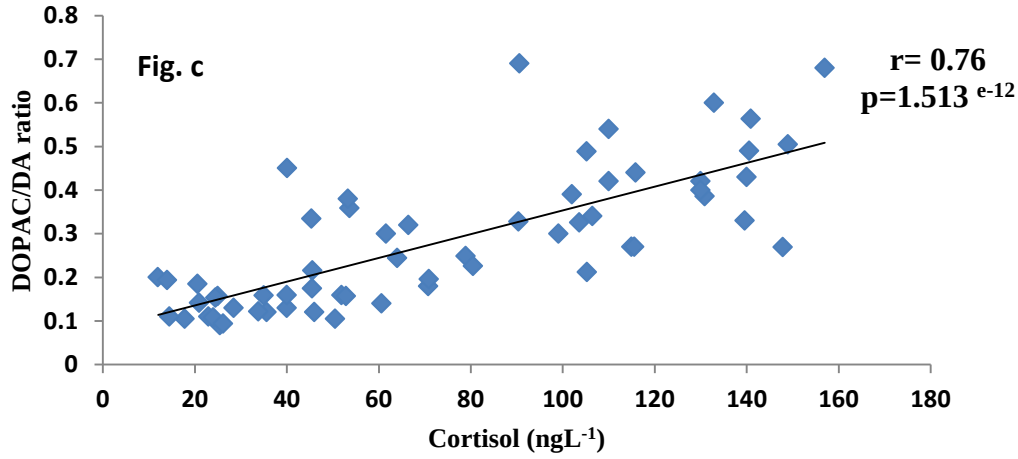


Fig. c
r= 0.76
p=1.513 e-12
DOPAC/DA ratio
Cortisol (ngL-1)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0
20
40
60
80
100
120
140
160
180

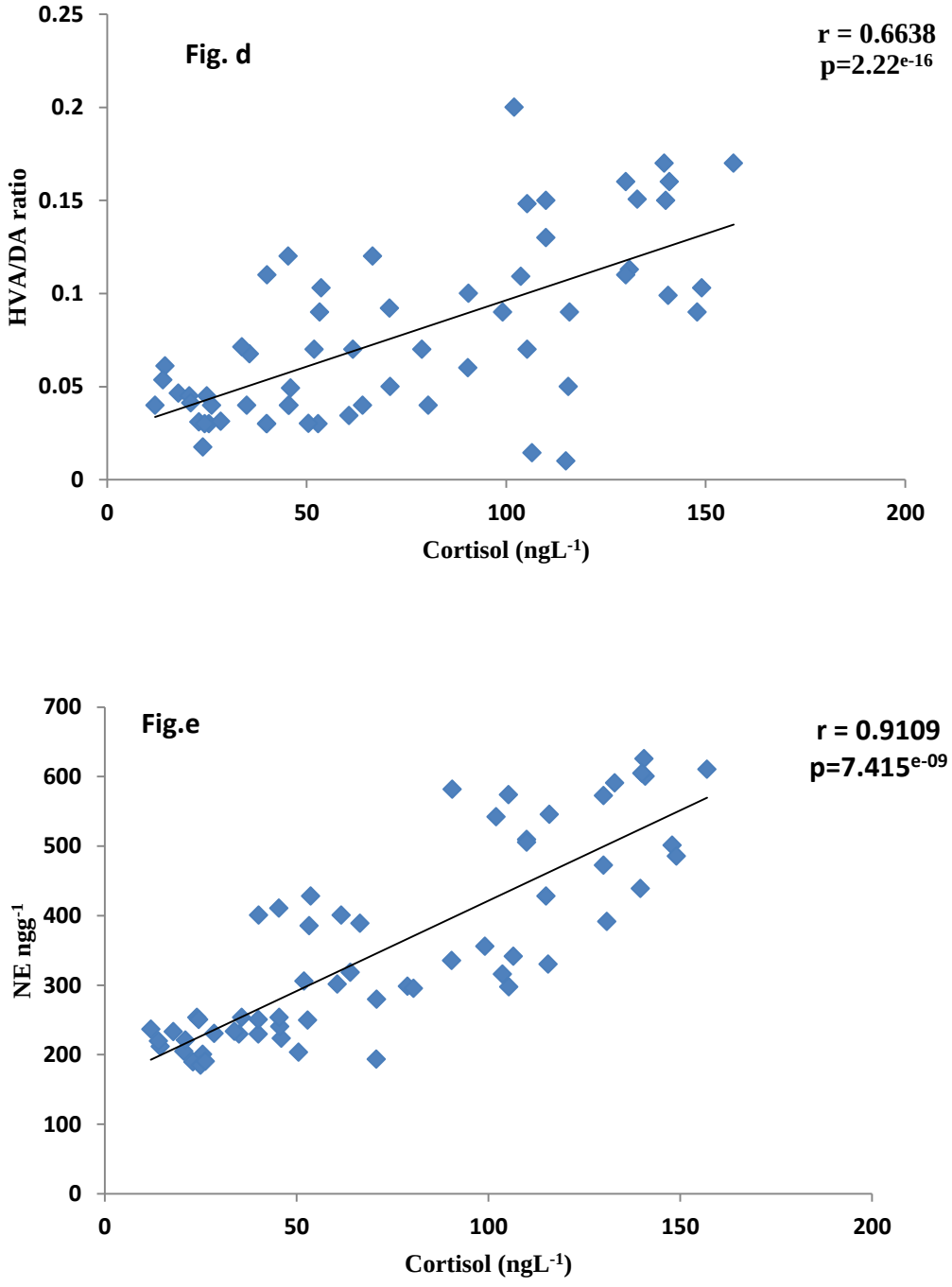


Fig. 8. The relationship of blood plasma cortisol with glucose (a) 5HIAA/5-HT (b) DOPAC/DA (c), HVA/DA (d), NE (e) in captive reared *T. putitora* subjected to acute stress. Pearson's correlation r and p values are given.

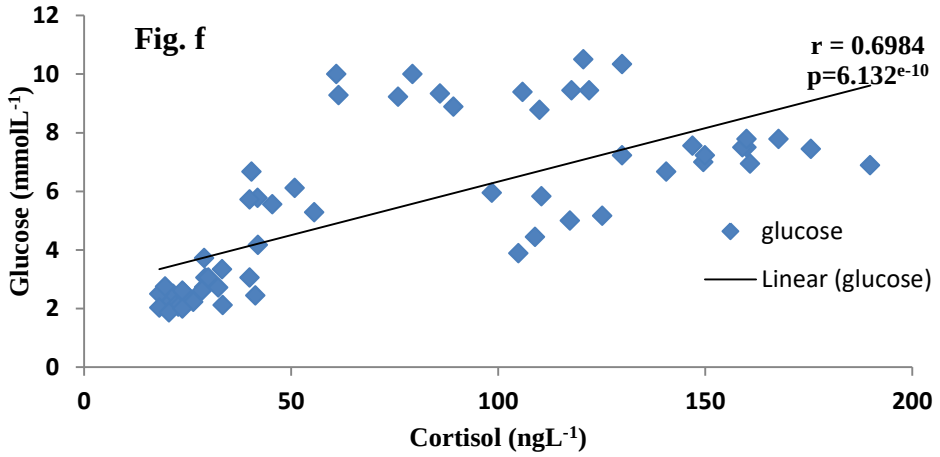


Fig. f
r = 0.6984
p=6.132e-10
Glucose (mmolL-1)
Cortisol (ngL-1)
glucose
Linear (glucose)
0
2
4
6
8
10
12
0
50
100
150
200

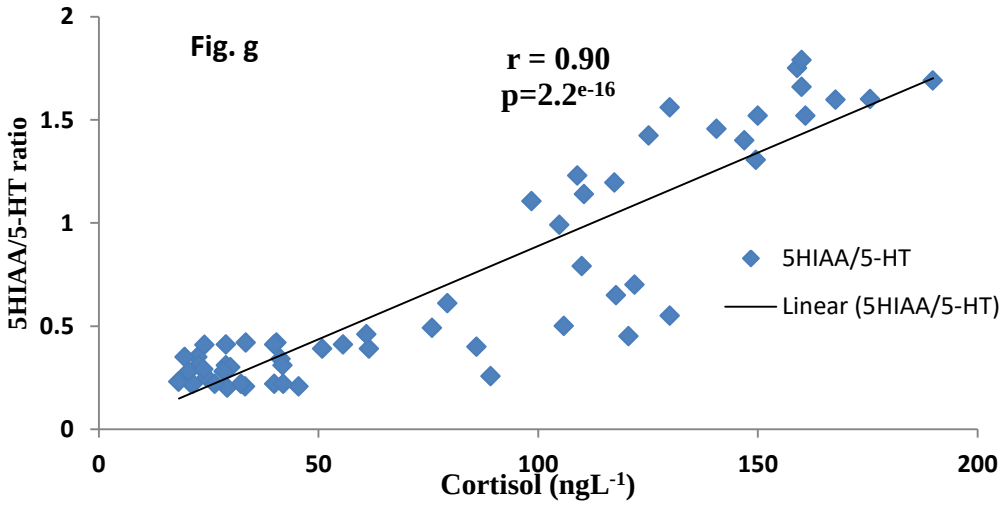


Fig. g
r = 0.90
p=2.2e-16
5HIAA/5-HT ratio
Cortisol (ngL-1)
5HIAA/5-HT
Linear (5HIAA/5-HT)
0
0.5
1
1.5
2
0
50
100
150
200

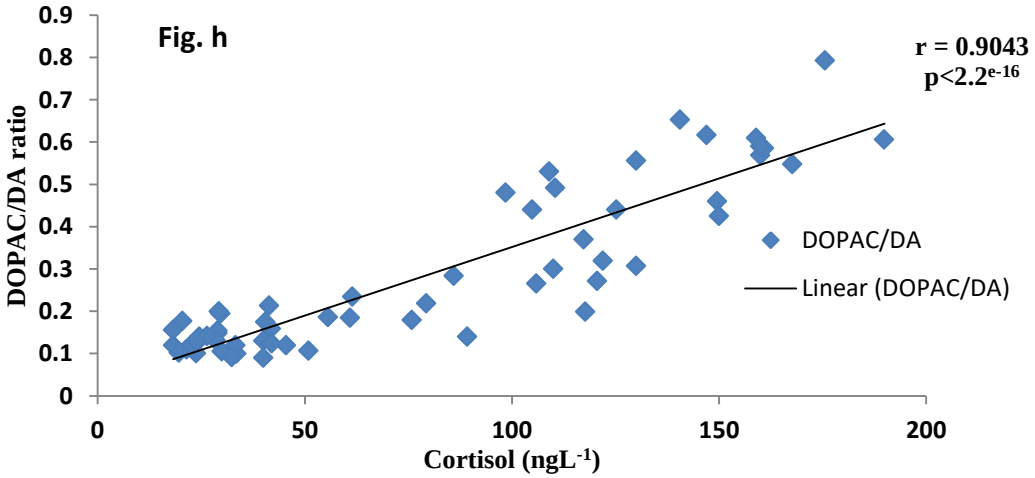


Fig. h
r = 0.9043
p<2.2e-16
DOPAC/DA ratio
Cortisol (ngL-1)
DOPAC/DA
Linear (DOPAC/DA)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
0
50
100
150
200

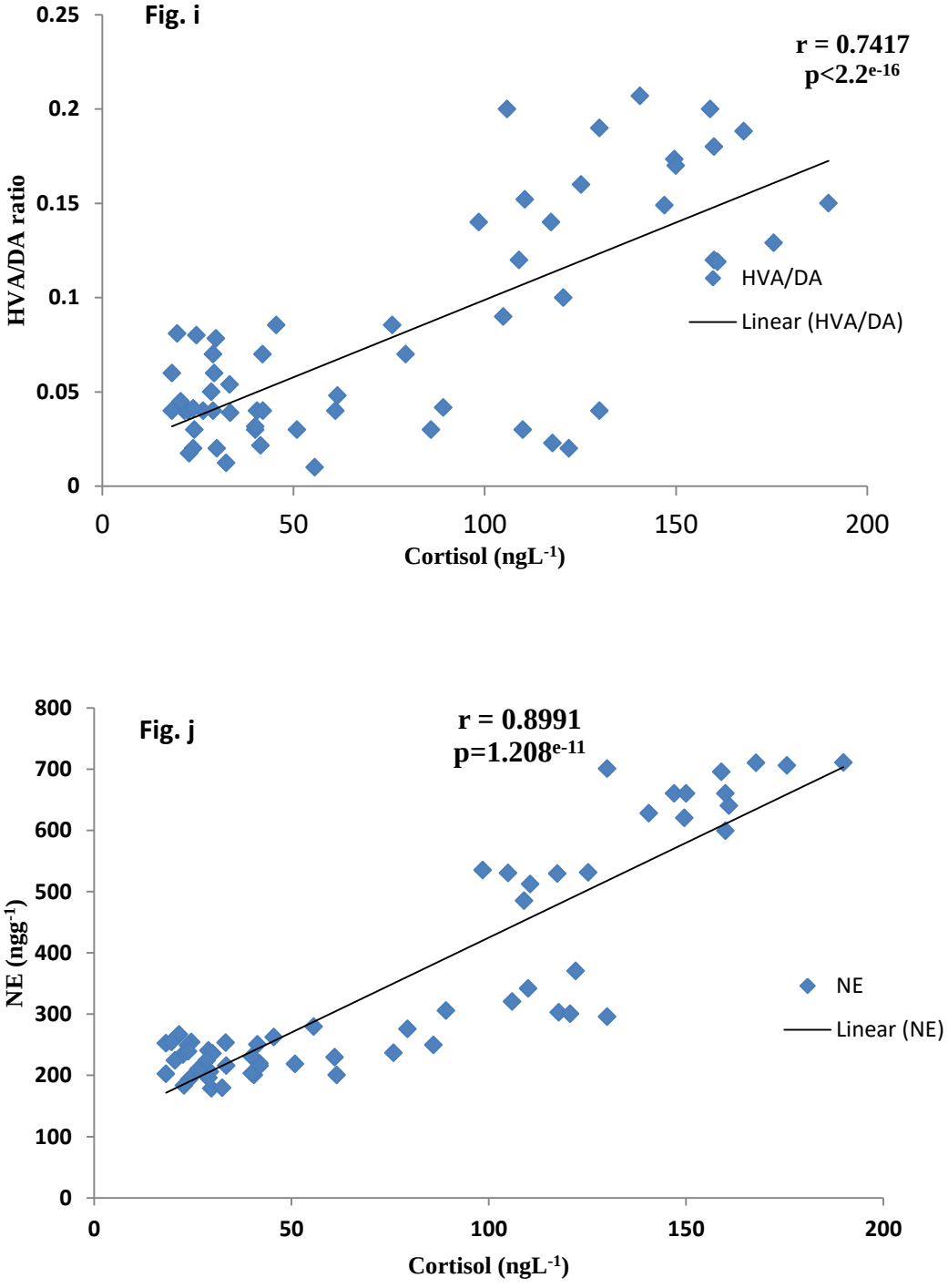


Fig. 9. The relationship of blood plasma cortisol with glucose (f) 5HIAA/5-HT (g) DOPAC/DA (h), HVA/DA (i), NE (j) in wild *T. putitora* subjected to acute stress. Pearson's correlation r and p values are given.

Table 15. Summary of relationship of plasma cortisol with brain monoamenergic activity (**monoamine/metabolite ratio)** and blood glucose in acute stress treated *T. putitora* from captive-reared and wild-caught population.

	Population	
	Captive	**Wild**
Glucose	r=0.80 p=1.288e-14	r= 0.69 p=6.132e-10
Monoamine/metabolite ratio		
5HIAA/5HT	r= 0.80 p=1.021e-14	r= 0.904 p=2.2e-16
DOPAC/DA	r= 0.76 p=1.513e-12	r= 0.90 p=< 2.2e-16
HVA/DA	r= 0.66 p=2.22e-16	r= 0.74 p= <2.2e-16
NE	r= 0.8298 p=7.415e-09	r= 0.8991 p=1.208e-11

Pearson's correlation r and p values are given, p value less than (0.05) show significant relationship. Relationship of blood plasma cortisol with monoamenergic activity and blood glucose are illustrated in figures 8 and 9.

Table 16. Summary of relationship of plasma cortisol with brain monoamenergic activity (monoamine/metabolite ratio) and blood glucose at different time interval in acute stress treated *T. putitora* from captive-reared and wild-caught population.

	Time (hr)									
Captive	Control	0.25h	0.5h	0.75h	2h	4h	6h	8h	24h	48hr
Glucose	r=0.16	r=-0.21	r=-0.64	r=-0.25	r=0.23	r=0.60	r=0.26	r=0.13	r=-0.16	r=-0.39
	p=0.16	p=0.68	p=-0.64	p=0.62	p=0.65	p=0.20	p=0.60	p=0.80	p=0.75	p=0.43
5HIAA/5HT	r=0.05	r=-0.16	r=-0.19	r=-0.14	r=-0.69	r=0.50	**r=0.90**	r=0.72	r=0.70	r=0.29
	p=0.70	p=0.74	p=0.46	p=0.78	p=0.12	p=0.30	**p=0.01**	p=0.10	p=0.11	p=0.56
DOPAC/DA	r=0.59	**r=-0.80**	r=0.46	r=0.08	r=-0.18	r=0.27	r=-0.20	r=0.52	r=-0.26	r=0.53
	p=0.35	**p=0.05**	p=0.61	p=0.87	p=0.7	p=0.59	p=0.70	p=0.28	p=0.60	p=0.27
HVA/DA	r=-0.07	r=-0.63	r=-0.43	r=0.16	r=-0.56	r=0.26	r=-0.48	r=-0.00	r=0.17	r=0.13
	p=0.38	p=0.17	p=0.73	p=0.7	p=0.24	p=0.60	p=0.32	p=0.98	p=0.73	p=0.80
NE	r=0.14	r=0.10	r=0.11	r=-0.49	r=0.40	r=-0.19	r=0.49	r=0.14	r=-0.12	r=-0.43
	p=0.82	p=0.84	p=0.21	p=0.31	p=0.42	p=0.70	p=0.32	p=0.78	p=0.81	p=0.38
Wild										
Glucose	r=-0.57	r=-0.13	r=0.35	r=0.66	r=0.52	r=0.43	r=-0.05	**r=0.87**	r=-0.61	r=0.36
	p=0.23	p=0.79	p=0.49	p=0.14	p=0.28	p=0.38	p=0.91	**p=0.02**	p=0.19	p=0.47
5HIAA/5HT	r=0.50	r=-0.36	r=0.006	r=0.67	r=-0.12	r=0.16	r=-0.16	r=-0.60	r=-0.43	r=-0.52
	p=0.30	p=0.48	p=0.99	p=0.14	p=0.81	p=0.75	p=0.75	p=0.20	p=0.39	p=0.28
DOPAC/DA	r=-0.29	r=0.17	r=-0.36	r=-0.29	r=-0.07	r=-0.13	**r=-0.83**	r=-0.01	r=0.03	r=-0.37
	p=0.57	p=0.7	p=0.47	p=0.57	p=0.88	p=0.80	**p=0.03**	p=0.97	p=0.94	p=0.46
HVA/DA	r=-0.478	r=-0.44	r=0.40	**r=0.88**	r=-0.46	r=-0.04	r=-0.24	r=-0.14	r=-0.35	r=-0.61
	p=0.33	p=0.37	p=0.42	**p=0.01**	p=0.35	p=0.93	p=0.64	p=0.78	p=0.48	p=0.19
NE	r=0.15	r=0.37	r=-0.02	r=-0.29	r=-0.26	r=-0.28	r=-0.09	r=-0.27	r=-0.00	r=-0.57
	p=0.77	p=0.46	p=0.95	p=0.56	p=0.61	p=0.58	p=0.85	p=0.59	p=0.98	p=0.23

Pearson's correlation r and p values are given, and significant relationships are indicated by bold font. p value less than (0.05) show significant relationship.

4. Discussion

The current results revealed that captive reared *T. putitora* showed subsequently different physiological stress response after being exposing common stressor i.e., 5 min chasing with hand net and 2 min confinement as compared to wild counterpart. Results of ANOVA of all physiological stress parameters clearly showed significant difference between two populations (Tables 1, 3, 5, 7, 9, 11, 13). Generally, wild population showed a rapid response to stressor and early recovery (Barton, 2002; Iwama et al., 2006; Zuberi et al., 2011) as compared to captive reared population which showed attenuated response. We are confident that differences in response between populations are due to altered developmental patterns of captive-reared population because the experiment was conducted in similar conditions and both populations were exposed similar stressor. Similar difference in stress response of captive and wild population have been reported already in many teleost species (Winberg et al., 2001; Perreault et al., 2003; Lepage et al., 2005; Zuberi et al., 2011; Gesto et al., 2013)

Cortisol is an extensively used indicator of stress (Barton, 2000; Fridell et al., 2007) and is often considered as an indicator of fish welfare (e.g. North et al., 2006; Turnbull et al., 2005; Varsamos et al., 2006), it rapidly increases in stressful condition (Zuberi et al., 2011; Barton, 2002; Wendelaar Bonga, 1997; Simontacchi et al., 2008; Wilkes et al., 2012) like netting, crowding, handling, live hauling (Vijayan et al., 1997; Arends et al., 1999) and exposure to predators (Zuberi et al., 2011). Cortisol secretion is the major end result of physiological stress response and it regulates energy metabolism (Wendelaar Bonga, 1997) and affect the fitness of the organism. Our results demonstrated a considerable difference in pre-stress and post stress plasma and water-borne cortisol levels in wild caught and captive reared mahseer (Table 2, 4, Fig.1, 2). Cortisol levels after exposure to stress confirm the view that wild fish showed a typical stress response that is rapid increased in plasma and water borne levels, i.e. at 0.75 and 2hrs respectively, after acute stress followed by rapid recovery, at 6 and 8 hrs respectively (Zuberi et al., 2011; Iwama et al., 2006; Barton, 2002) while captive reared mahseer showed atypical response. The cortisol level in the captive population increases slowly and showed a continuously increasing trend even after 4 (Plasma) and 6 hrs (Water-borne). The

experiment was conducted in replicate under the similar environmental condition by using similar stress stimulus; therefore we are certain that variation in response appeared due to physiological difference of both populations.

The results of acute stress response of wild mahseer were similar to those observed in other fish species where the stress response normally appears between 30 min and 4 h after exposure to a stressor (Zuberi et al., 2011; Barton, 2002; Scott et al., 2008). For example, wild caught rainbow fish (*Melanoteania duboulayi*) showed a marked increase in the cortisol release rate within 30 min after exposing stress by chasing with a simulated predator (Zuberi et al., 2011) and showed a recovery trend within 4 hrs similar to observed in case of wild mahseer population compared to captive reared mahseer. Similarly, in rainbow trout, *Oncorhynchus mykiss* Walbaum plasma cortisol levels peaked within 30 min after the onset of mild confinement stress (Pottinger and Moran, 1993) while water cortisol level peaked at 2 hr post mild handling stress followed by recovery (Ellis et al., 2004). Moreover, in carp, *Cyprinus carpio* L. cortisol levels returned to basal levels after 4 hr of exposure to stress by capturing and holding in nets (Pottinger, 1998).

In acute stress the release of cortisol is temporary and it provides energy to cope with the environment, while the prolong release of this hormone resulted in adverse effects. The present study showed that in captive reared mahseer, water borne cortisol level at 2 hr was 44.91 ± 91 μgL^{-1} statistically comparable to peak level (46.48 ± 3.32 μgL^{-1}) at 4 hrs, then showed decreasing trend (Fig.1, Table. 2). This result is somewhat different with the stress response of captive reared *Melanoteania duboulayi* (Zuberi et al., 2011) where water-borne cortisol increased after 4 hr of stress exposure and remained at elevated levels. It may be due to the captive bred generation used for experimental purpose. We used fourth generation of *T. putitora* as compared to fifteenth generation of *M. duboulayi*. However, wild population of *T. putitora* showed a similar trend as reported for wild *Melanoteania duboulayi* (Zuberi et al., 2011) i.e. attainment of peak levels at 2 hrs post stress and rapid recovery. Our results of gradual increased in water-borne cortosl for an extended period in captive reared mahseer further showed the odd physiological response of hatchery reared fish.

Physiological stress response and cortisol levels after experiencing a stressful event is heritable factor (Fevolden et al., 1991; Fevolden and Roed, 1993; Pottinger et al., 1994; Fevolden et al., 1999), while stress responses heritability (h^2) may vary noticeably in population and species under consideration. Many scientists suggest that even a single generation under hatchery conditions can showed profound effect on the behaviour of fish (Álvarez and Nicieza, 2003; Salonen and Peuhkuri, 2006) while repeated stressors can increase the heritability of plasma cortisol (Fevolden et al., 1999). In hatchery-reared Atlantic salmon and rainbow trout heritability value of stress response were 0.05 and 0.27 respectively, while it increased to 0.56 in rainbow trout after repeated stressors (Fevolden et al., 1999). Moreover magnitude of corticosteroid response is different among species and depends upon the duration and severity of the stressor, environmental conditions and developmental stage of fish (Ellis, 2004; Barton, 2002).

In fish bile, urine and gills are three main ways through which free steroids are cleared from plasma and released into the water (Vermeirssen and Scott, 1996; Sorensen et al., 2000). Mostly cortisol is released from the anterior region of the gills (a passive 'leakage') due to concentration gradient among plasma and surrounding waters (Scott et al., 2002). Like our methodology, many investigators adopted both invasive (From blood) and non-invasive (through water) techniques for examining the difference in corticosteroids response to stressors in various fish species (Ellis, 2004; Barton, 2002).

In the present study, we have been using both invasive and non-invasive approaches to screen the possible effect of rearing environment on the physiology of fish. The plasma level of cortisol in a wild mahseer was 167 ± 0.04 ngL^{-1}, significantly ($P < 0.05$) higher than observed in captive reared mahseer (135.23 ± 5.84 $ngmL^{-1}$) (Fig. 2, Table 4). These findings support over non-invasive results because it is well documented that in fish water-borne cortisol level correlated with the plasma cortisol concentration (Ellis et al., 2004). Furthermore, the results further provide evidence of the negative impact of the captive rearing environment on the behaviour and stress response of fish.

In addition to enhancing the concentration of blood cortisol, stress typically elevate plasma glucose levels (Pottinger et al., 2000) and this is initially boosted by catecholamine-mediated glycogenolysis and later on by cortisol-mediated

gluconeogenesis (Begg and Pankhurst, 2004; Mommen et al., 1999; Guesto et al., 2016). Cortisol and glucose are the reliable indicator of fish stress and increase level of glucose indicate the demand of energy arise to cope with the stressful situation (Martínez-Porchas, et al., 2009). Post-stress increases in plasma glucose are sometimes used as alternate for activation of the HPI axis. Rises in plasma glucose may be limited in species with limited hepatic glycogen stores (Pottinger et al., 2002; Wright et al., 2007) or show a different increase and recovery profiles from cortisol (Pickering et al., 1982; Pottinger, 1998; Flodmark et al., 2002).

In current observation, we find no difference in basal levels of blood glucose in both populations. However, we demonstrate here the typical increase blood glucose in response to acute stress in wild mahseer compared to captive reared one (Table 6. Fig 3). In current findings, blood glucose level showing an increasing trend within the initial 15 min post stress, while peak level were observed at 2 and 4 hr post stress and then gradually decrease from 4 hr to 48 hr after stress. In the available studies, plasma glucose usually returned to basal levels in a few hours, although recovery times strongly depend on the species, the kind of stressor and exposure time (Biron and Benfey, 1994; Wilson et al., 1998; Arends et al., 1999; Small, 2004; Aluru and Vijayan, 2006; Fast et al., 2008). Similar to cortisol, the glucose levels were also returned to pre-stress values but had taken longer time period about 24 hrs (wild population) and 48 hrs (Captive reared) after acute physical stress compared to cortisol (Fig. 3) . Similar higher time to recover a normal level was also reported in other teleost fish (Bracewell et al 2004; Jentoft et al., 2005; King and Berlinsky, 2006; Fast et al., 2008). It appears that like previous observations, blood glucose profile in both populations did not match the cortisol profiles (Pickering et al., 1982; Pottinger, 1998; Flodmark et al., 2002). As blood glucose levels of mahseer increased in two phases, an initial fast increase occurred in less than 0.5 h, and is most probably the result of an increase glycogenolytic process in liver elicited by enhanced levels of central and blood catecholamines (Guesto et al., 2016). A second increase was observed between 45 min and 2 hrs to 4hrs, which could be related to the secondary effects of cortisol promoting gluconeogenesis in the liver (Mommsen et al., 1999). Similar observation reported by Guesto et al. (2016) in rainbow trout that after 5 min chasing stress plasma cortisol increased in two phases. Furthermore, captive reared

mahseer showed lower level of glucose at all time periods after stress as compared to wild counterpart (Table 6, Fig. 3).. This difference in the magnitude of the glucose and recovery time may be due to differences in previous rearing environment.

It has been reported that when fish confront a stressful condition brain serotonin (5-HT) and catecholamine (DA and NE) systems are also activated (Winberget al., 1992; Papoutsoglou et al., 2006; Karakatsouli et al., 2007). The elevation of 5-HIAA/5-HT and DOPAC/DA ratios in several brain areas and up-regulation of 5-HT synthesis appears to be an important mechanism to cope with stress (Lepage et al., 2000). Backström et al. (2011) observed increase concentrations of brain 5-HT, 5-HIAA and HVA and HVA/DA ratios in response to confinement. Furthermore, Øverli et al. (1999) observed a gradual increase over time in monoamine metabolites in brain stressed fish. Like others, we also observed increase levels of brain 5-HT/5-HIAA, DOPAC/DA, NE and HVA/DA in response to acute stress (5 min chasing and 2 min confinement) suggesting that a stress-induced activation of brain dopaminergic and serotonergic systems. However, we did not find any significant variation in basal levels of 5HIAA/5HT, DOPAC/DA, NE and HVA/DA ratios between captive reared and wild caught *T. Putitora.*

Acute stress caused a rapid activation of dopaminergic and serotonergic systems in both populations (Captive reared and wild caught mahseer) indicated by elevated levels of 5-HT/5-HIAA, DOPAC/DA, NE and HVA/DA ratios (Tables 8, 10, 12, 14 Figurs 4, 5, 6, 7).. The significant ($P < 0.05$), two way interactions between population and treatment of HT/5-HIAA ratio (Table 7) and NE (Table 13) revealed the difference in physiological stress response of both populations. The effect of stress on the increasing trend of serotonergic activity was transient, and serotonergic ratio returns back to control levels in a few hours (4 to 6 hr; Fig. 4). Although, like blood cortisol and glucose, serotonergic activity also showed recovery, but in the quick way, i.e. within 4 (wild population) to 6 hr (captive reared mahseer) as compared to 8 to 24 hrs (Fig. 4) . The observed peaks in serotonergic activities at 0.75 h after stress could be the result of the indirect effects of other participants in the stress response such as cortisol, which is

known to promote serotonergic activation fish (Gesto et al., 2016; Summers and Winberg, 2006; Weber et al., 2012).

Brain serotonergic systems influence cortisol secretion under normal and during moderate or short-term stress (Overli et al., 1999). It appears that brain 5-HT could also play an important role in the regulation of the HPI axis (Winberg et al., 1997). In the mammalian brain, 5-HT stimulates the release of CRH, which in turn activates corticotrops of the pituitary (Chaouloff, 1993; Dinan, 1996). There is also an evidence of direct action of 5-HT on corticotrops of the mammalian pituitary (Chaouloff, 1993; Dinan, 1996). Although the influence serotonergic systems with the release of cortisol is not well studied in fish, but Winberg and Nilsson (1993) suggested a relationship between HPI axis and the brain 5-HT system and reported dose dependent elevation of plasma cortisol after treatment of rainbow trout (*Oncorhynchus mykiss*) with a potent 5-HTIA receptor agonist, 8-hydroxy-2-(di-n-propylamino)-tetralin (8-OH-DPAT) (Winberg et al., 1997). We also find positive correlation between plasma cortisol and brain monoamines (Table 15, 16 Figurs. 8, 9) and suggest the influence of monoamines in the regulation of HPI axis. In fact, the involvement of serotonergic activity in the stress response is very complex and in mammals 5HT probably stimulates CRF release by the hypothalamus (Boisvert et al., 2011; Calogero et al., 1989) and ACTH release from the pituitary (Calogero et al., 1993). However, in fish, no such study is available.

In mammals, both stimulatory and inhibitory effects of stress on dopaminergic activity have been reported (Höglund et al., 2001; Waters et al., 2005). In fish, several studies have reported a stimulatory action of different kinds of stressors on the central dopaminergic system (Backström et al., 2011; Gesto et al., 2008; Øverli et al., 1999; Weber et al., 2012). Lepage et al., (2000) reported differences in post-tress midbrain DOPAC/DA ratios between wild and domesticated fish. Domesticated fish displayed lower post-stress brain 5-HIAA/5-HT and DOPAC/DA ratios than wild trout (Lepage et al., 2000). In current study DOPAC/DA ratio increased after stress in the brain of mahseer in both populations supporting the idea that the activation of the dopaminergic activity due to acute stress.However, DOPAC/DA as well as HVA/DA did not show significant interactions between population and treatment ($p = 0.48$, Table 9). It appears

that these parameters are not reliable indicators to differentiate populations on the basis of small physiological difference. Captive reared and wild caught mahseer, although showed statistically different physiological stress response with respect to blood and water-borne cortisol, blood glucose, 5HIAA/5-HT and NE, but except NE all parameters did not show highly significant interactions between population and treatment. It may be due to interbreeding of wild and captive reared fish because of restocking program or due to the small genetic distance between population as we have used 4th generation captive reared fish.

In the present study, the increase in concentration of NE after stress in both populations indicated the involvement of the brain noradrenergic system. Like our results, Øverli et al. (1999) also reported the rapid activation of brain norepinephric (NE) systems in rainbow trout after acute stress. It has been reported that the NE system involve in the stress response in mammals (Dunn et al., 2004) and probably in fish (Øverli et al., 2001). Although both captive reared and wild mahseer had a similar basal level of NE but the exposure of acute stress showed a significantly higher level NE at different time interval in a wild population, compared to captive reared counterpart indicating the difference in physiological stress response of both populations which may be due to captive rearing divergence of hatchery reared fish.

Conclusion

The present study has demonstrated that wild-caught mahseer demonstrate different stress response compared to the captive-reared counterpart. These findings suggest that captive rearing can lead mahseer in the physiological divergence from wild fish. The physiological stress response, which compared the blood plasma (invasive) and water-born (non-invasive) cortisol, blood glucose and brain monoamines of wild mahseer with that of their captive-reared counterparts, revealed differences in HPI-axis the level of stress response. Thus the outcomes of this study suggest a modification of the hatchery rearing environment in such a way that will produce fish, physiologically comparable to their wild counterpart. These findings have important implications for a possible way of

improving the welfare of mahseer and in the long term improve the conservation of this endangered species.

Chapter # 2

Evaluation of life skills behaviour of Captive-reared and Wild-caught Endangered Fish Mahseer (*Tor putitora*)

ABSTRACT

Release of hatchery reared fish in the natural environment is one of the most important strategies to replenish the natural stock of endangered fish species. Most of the reintroduction projects are not providing the desired results, since captive-reared fish may not possess the behavioural skills required for survival in the natural environment. Behaviour differences between captive and wild caught fish have frequently been observed. Here an attempt has been made to evaluate variation in the life skill behaviours of captive-reared and wild-caught endangered fish mahseer (*Tor putitora*). Under laboratory trials, boldness, exploratory, predatory and anti-predatory behaviour of both populations were compared. The initial behavioural descriptions of mahseer showed that wild fish display significantly ($p < 0.05$) more exploratory, predatory and anti-predatory behaviour in comparison to the captive reared fish. However, captive mahseer appeared bolder as compared to their wild counterpart. These results specify the role of rearing environment in shaping the behaviour of fish and suggest an improvement in the already existing rearing environment, to reduce the behaviour deficiencies and increase the post survivourship of captive reared mahseer.

1. Introduction

Release of captive reared animals in the natural environment is one of the most important strategy for the conservation of severely depleted populations (Myers et al., 2004; Braithwaite and Salvanes, 2005; Araki, et al., 2008) and replenishment of fishery stocks to the levels that can support sustainable fisheries (Bell et al., 2006; Lorenzen, 2005). These programs are generally used to mitigate the effects of over-fishing, recruitment failures and environmental degradation (Myers et al., 2004). However, the success rate of such programs would be based on both survival and fitness of the released fish in their natural habitat. Several studies reported the low survival of newly released hatchery reared fish in many such restocking practices (Olla et al., 1998; Ellis et al., 2002; Araki et al., 2008), may be due to poor performance after the release in the natural environment (Le Vay et al., 2007; Garlock et al., 2014).

Rearing conditions strongly affect the ability of the fish to acquire important life skills, such as predator recognition and avoidance (Yamamoto and Reinhardt, 2003; de Azevedo and Young, 2006), exploratory, predatory, foraging behaviours (Johnsson et al., 2014), boldness (Kelley et al., 2006) and reproductive behaviour (Kelley et al., 2006; Berejikian et al., 2009). The captive environment differs from the wild in complexity of habitat, food availability, stocking densities and human intervention (Huntingford, 2004). Consequently, captive animals, encounter experiences different compared to wild counterpart. Several studies on hatchery reared fish reveals that, lack of appropriate variation (physical, social etc.) in the rearing environment, influences the phenotype ranging from physiology to behaviour (Huntingford, 2004; Brännäs and Johnsson, 2008).

Captive rearing plans fail to raise individuals with natural behaviour (Araki et al., 2008), affect the life-skills training and produces behaviorally deficient individuals, lacking the skills to cope the challenges of wild environments (Brown and Day, 2002; Huntingford, 2004; Bell et al., 2006; Le Vay et al., 2007; Araki et al., 2008; Garlock et al., 2014; Johnsson et al., 2014), hence affecting the success of reintroduction strategies (McPhee, 2003).

In captivity, at hatcheries, fish are typically grow in static, featureless environments at high densities and in an excess of pellet food, thus preventing them from competition and learning how to capture natural live prey, while wild fish which live in more complex environments learn by previous exposure (Sundström and Johnsson, 2001; Huntingford, 2004). Moreover, due to the absence of predators, fish in captivity do not trade off between risk of predation and other activities like foraging opportunities and courtship behaviour (Huntingford, 2004). Several studies have demonstrated that high stocking density in the captive rearing environment promotes aggression (Kaiser et al., 1995; Kelley et al., 2006). Aggressive behaviour is often correlated with other traits such as boldness and bold individuals seek less refuge and take greater risks to exploit the resources (Ward et al., 2004; Kelley et al., 2006). Generally boldness is associated with life skill activities like foraging and exploration, anti-predator behaviour, mate selection, learning ability, etc. (Brown et al., 2007a; Wilson and Stevens, 2005) and is correlated with fitness-related traits (Sinn et al., 2006; Brown et al., 2007b). Hence, captivity strongly affects the ability of fish to acquire important life skills and raise questions to the validity of stock supplementation from hatchery reared fish (Brown and Day, 2002; Huntingford, 2004; Trushenski et al., 2015).

Early life experiences and rearing environments shape an animal behaviour and personality (Huntingford, 2004; El Balaa and Blouin-Demers, 2011). The hatchery selection favours fish that are well adapted to captivity, but maladaptive to the wild leading to differences in behaviour, physiology and survival (Christie et al., 2012). Investigating the influence of captive rearing and evaluating the behaviour of wild and captive animals is of particular importance in many contexts, including conservation biology, behavioural ecology and captive breeding programs to restore the wild populations (ElBalaa and Blouin-Demers, 2011).

Mahseer is among the most important semi-cold freshwater fish of Pakistan and South Asian countries. It has great importance as food and for sport fishing (Khajuria and Langer, 2016). However, its population in the wild environment is reported to be declining due to several human and environmental factors and therefore, it is declared as an endangered fish (IUCN, 2015). Fish scientists emphasize special attention to protect this fish species from future extinction (Bhatt et al., 2016).

The Pakistan government has also realized the economic importance of mahseer, and has taken steps for their conservation. Hatcheries have been established in the Punjab, Azad Jammu and Kashmir (AJK) and Khyber Pukhtunkhwa (KP) for the artificial propagation of this species. Moreover, for replenishment of the river and other natural bodies, restocking program has been initiated that involve the release of artificially propagated hatchery reared fish in natural bodies. In spite of ample efforts, the results are not encouraging and captures of this species from natural reservoirs is continuously declining (Personal observation and communication with Punjab Fisheries, AJK fisheries and KP fisheries departments). It seems that hatchery reared mahseer in the natural environment underwent heavy mortality, which may be due to behavioural deficiencies.

We predicted that distinct captive and wild environments will generate differences in many aspects of behavioural skills of mahseer, through differential exposure during early development and rearing. Therefore, we designed an experiment to study the behaviour of wild-caught and captive-reared endangered fish and anticipated that the results will help to understand the impact of rearing environment in shaping the behaviour, as well as help to develop a proper culture system that could promote the welfare and conservation of this species. Quantified behavioural traits and life-skills of wild and hatchery reared fish will further be associated with the success of release programs of hatchery reared mahseer into their natural environment.

2. Materials and methods

2.1. Fish collection from wild environment

Wild caught mahseer (mean ± SEM body mass and length 15.5±0.37g; 9.2 ±0.29 cm) were captured through drift gill net (size, 25m L× 4m W; mesh size, 2cm) from River Haro, Attock 33°46'8" N, 72°14'43" E in DMS (Degrees, Minutes, Seconds) and transported alive (3 g/L) in oxygen filled plastic bags (36 cm L × 24cm W; 25 L water) to the Fisheries and Aquaculture Research Center, Department of Animal Sciences, Quaid-I-Azam University Islamabad, Pakistan. Fish were initially housed in replicates of three (with a stocking density of 1.5 g/L: 35 fish/tank) in well aerated circular fibreglass tanks (volume, 500 L water) containing 1cm deep gravel (black and white colour) substrate, river stones (5-6 cm diameter) and artificial plastic plants (25 cm height: n = 10) for at least two weeks before any further experimentation. Temperature and photoperiod were maintained at 22.5 °C and 12:12 h light and dark respectively. Water quality in terms of total ammonia (< 0.25mg/ L), pH (from 6.8 to 7.48) and dissolved oxygen (> 5.2 to 5.6 mg/L) were found to be in the acceptable range for mahseer. During acclimation period, wild caught fish were gradually weaned from live food onto prepared pelleted feed (sinking pellets; Oryza Organics fish feed, size 2 mm; 45 % crude protein, 14 % fats, crude fibre 2 % and 10 % moisture. Fish were fed 4 % body weight twice daily (9:00 h and 17:00 h).

2.2 Fish collection from captive environment

2.2.1 Conventional breeding and rearing of Mahseer

In Pakistan, artificial propagation of mahseer in captivity was started in 2001 at Mahseer Fish Hatchery Hattian Attock, Punjab, Pakistan. Generally, at hatchery, fish brooders are prepared in structureless concrete rectangular brooders tank (50×50 ft) on prepared pelleted feed. Hand stripping is the method in practice for obtaining eggs and sperms. The fertilized eggs are spread on the fibreglass hatching tray (size, 1.5 ft W × 15 ft L) and are placed in a hatchery room under direct water sprinkling. After hatching (70 to 100 hrs depending upon water temperature), the hatchlings are shifted to barren circular concrete tank (7 ft diameter × 4 ft H). After semi-quiescent

stage (yolk sac absorption), when fry starts exogenous feeding, they are relocated in semi-earthen ponds (50 W × 100 L ft), earthen bed with concrete walls, having natural food organisms (phytoplankton, zooplankton, diatoms, some protozoa etc.) but without common aquatic weeds or other substrate. They are raised up to fingerling stage on live feed, plus pelleted feed by adopting monoculture technique or in combination with other species like common carp (*Cypriuns carpio),* rohu *(Labeo rohita*).

Breed of the fourth generation, captive reared mahseer (mean ± SEM body mass and length 15.2 ± 0.58 g and 8.5 ± 0.23 cm) was collected from the hatchery and also transported to the Fisheries and Aquaculture Research Center. The fish were acclimated for at least 2 weeks in well-aerated circular fibreglass tank (volume, 500 L water) with same stocking density as used for wild fish, before starting the experiments. The fish were maintained under a controlled photoperiod (12:12 h day: night) and temperature (22.5 °C). Water quality parameters were maintained at optimum level.

2.3. Behavioural Study

2.3.1. Temperament assay

2.3.1.1. Assessment of boldness: Motivation to leave shelter

The boldness assay was performed by following procedure reported previously (Ullah et al., 2017) for the same species. Briefly, a start box (30 cm L × 60 cm W × 60 cm H) having door in the centre of one wall was set in test aquarium (90 cm L × 60 cm W × 60 cm H) that three sides were covered with black plastic for avoiding outside disturbances. Fish from experimental aquarium were transferred by a hand net (30 cm × 30 cm) to the start box. An individual fish was placed into the start box and provided 2 min settling period (a standard length of time in such experiments). After settlement, a door of the start box was raised remotely by using a fine monofilament and fish were timed until they emerged fully from this start box. Those fish had not emerged after 10 min, were given a maximum score (10 min) of emerging time while those emerged sooner were considered to be bolder.

2.3.1.2. Exploratory behaviour

Exploratory behaviour of mahseer reared in wild and captive environments was studied by method reported previously (Ullah et al., 2017). Briefly, the test aquarium was divided into three habitats by setting plastic plants on one quarter left hand side and a white, non-transparent plastic "start box" (30 cm L × 60 cm W × 60 cm H) on another one quarter right-hand side of the aquarium, while the area between the start box and the artificial plastic plants, were kept open.

One fish from each group was placed in the start box and allowed to familiarize with the environment for 10 min. After that, the door (30 cm W × 30 cm H) in the centre of one wall of the box facing the open area and vegetation area was slowly open by lifting the door in the centre of the box. The door of the start box was remained open until the end of the trial and measured the time spent by fish in the start box area. If the fish had not left the box after 30 min, the test was ended. If the fish had left the box, its position in the aquarium was recorded after every 30 sec for 10 min. The number of habitat shift between box, open and vegetation area was also recorded for each individual fish during the trial. For evaluation of behavioural difference, ten repeats (n=10) for each wild caught and captive reared population were performed, with a new fish in each trial.

2.3.2. Feeding on live prey

Predation of wild caught and captive reared mahseer was studied according to the method adopted by Ullah et al. (2017). Briefly, three sides of the test aquarium (90 cm L× 24 cm W × 24 cm H) were covered with black plastic sheets, leaving the long end facing the video camera uncovered. The bottom of the tank was covered with 1 cm gravel bed with a plastic plant (25 cm H; n = 4) in one quarter. Since mahseer prefers to feed in groups (Desai, 2003), five fish (n = 5 shoals per trial) from the wild or captive population were placed in an aquarium where earthworm were present at a density of 2 earthworm per litre. To enhance the feeding motivation, the mahseer was fasted for 24 h before feeding trial. The time taken until the first attack on prey was recorded for each fish in the group. Ten trials per population (wild and captive) were run with five new fish in each

trial. The trial ended when all fish attacked prey at least once, or after 120 min. The feeding behaviour of fish (Table 1) was recorded by was recorded by video camera connected to a computer was placed in front of the longe uncovered side of the experimental tank as previously described by (Ullah et al., 2017) for the same species.

Table 1. The Behaviour of mahseer observed during feeding on live prey (earthworm) trial (Ullah et al., 2017).

Behaviour	Description
Inspection	Mahseer swims towards and observe the prey
Picking	Mahseer picks up the live prey and suddenly leaves them and rapidly move away from prey
Feeding	Consumption of prey

2.3.3. Anti-predator response

Wild and captive reared mahseer were exposed to predator catfish Malli (*Wallago attu*) to monitor the anti-predatory response of mahseer. Two equally sized glass aquaria (90 cm L × 60 cm W × 60 cm H) were placed next to each other, separated by a removable cardboard divider to prevent visibility between the aquaria. One aquarium contained a freshwater predatory catfish at least doubles the size of the of mahseer placed in the other aquarium. The two short ends of the test aquarium containing the catfish were covered with black plastic sheets, with the long ends facing the mahseer and the observers uncovered. Three sides of the aquarium containing the mahseer were covered, with the long side facing the catfish aquarium uncovered. The bottom of the tank was covered with 1 cm gravel bed. In the left hand side corner of the mahseer aquarium, plastic plant was offering a possible refugee occupied approximately one third of the aquarium. The catfish and mahseer were allowed to acclimatize in the test aquarium for one hour before the tests started. After 30 min, the divider was carefully removed, not to startle the fish, allowing visual contact between mahseer and catfish. The feeding behaviour of fish (Table 2) was recorded a video camera connected to a computer placed in front of the longe uncovered side of the experimental tank during the

procedures every 30 sec during 10 min. For each population, 10 trials were run, with one new fish in each trial.

Table 2. Behaviour observed during anti-predator response trials (Ullah et al., 2017)

Inactive Behaviour	
Freeze	Lying motionless on the bottom of the experimental tank
Hide	Motionless or low activity within the refuge
Low activity	Slow movement outside the refuge
Active Behaviour	
Inspection	Swims toward the predator /larger fish
Move away	The mahseer moves directionally away from the predator
Skitter	Rapid movements with frequent changes of direction

2.2. Statistical analysis

The results are expressed as mean ± SEM.. Statistical analysis was carried out by using lme4 (Bates et al., 2014) and easynova (Arnhold 2013) package of R 3.2.5 (R Development Core Team, 2016). The assumption of normality, homogeneity of variances and additivity of the model were checked by Shapiro-Wilks, Levene's and Tukey 1-dF Test respectively. The effects of changing environment on exploratory, predatory and anti-predatory behavioural responses were analysed by ANOVA in factorial and split plot by using ea2 command of R with double factorial in completely randomized design followed by post hoc *Tukey's HSD*. The data of boldness and habitat shift were analysed by using Welch two sample t-test. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Temperament assay

3.1.1. Boldness Assessment: Latency to leave shelter

There was a significant difference in latency to leave the shelter among two different populations. Captive reared fish preferred to leave the box area sooner than the wild caught fish (n = 10, mean ± SEM: 5.04 ± 0.55min and 7.13 ± 0.89 min, respectively, t = -2.51, $p<0.01$; Fig. 1).

3.1.2. Exploratory behaviour

Exploratory behaviour of both populations showed significant difference ($p < 0.001$) (Table 3, Fig. 2). The proportion of time spent in box area between captive reared and wild caught mahseer was significantly different (n = 10, mean ± SEM: 32.23 ± 3.65 min and 19.84 ± 2.67 min, respectively; Tukey's post hoc, $p <0.001$). Similarly, time spent in the open area also showed significant differences (n = 10, mean ± SEM: 46.12 ± 5.00 min and 28.69 ± 2.81 min respectively, Tukey's post hoc, p = 0.001). Wild caught as compared to captive reared fish preferred to spend more time in the area having plants (n = 10, mean ± SEM: 51.47 ± 4.32 min and 21.68 ± 1.66 min, respectively, Tukey's post hoc, p = 0.001). Fish of both populations also showed significant differences in number of habitat shifts, i.e. movement between three different areas of aquaria, box, open and vegetation (n = 10, mean ± SEM: 5.34 ± 0.47 min and 7.63 ± 0.59 min respectively, t = 3.02, $p < 0.04$; Fig. 3).

3.3. Live prey test

In live prey tests, a significant difference was observed among populations (Table 3, Fig. 4). Wild caught and captive reared populations showed significant difference in live prey inspection (n= 10, mean ± SEM: 45.17 ± 3.66 min and 23.88 ± 1.36 min, respectively, Tukey's post hoc, p = 0.001), Picking time of live prey (n = 10, mean ± SEM: 76.22 ± 5.04 min and 30.55 ± 2.71 min, respectively, Tukey's post

hoc, p = 0.001) as well as feeding time on live earthworm (n = 10, mean ± SEM: 88.92 ± 7.81 min and 38.77 ± 3.92 min, respectively, Tukey's post hoc, p < 0.001).

3.4. Anti-predatory behaviour

Anti-predatory behavioural response of both populations showed significant difference (Table 3, Fig. 5). The captive reared fish performed significantly more inactive behaviours (freeze, hide), than wild caught fishes. However, low activity is an inactive behaviour which is significantly higher in wild reared fish. When comparing the proportion of time spent performing behaviours separately, a significant difference between the two populations in all anti-predatory behaviours (active and inactive response) was observed. There was a significant difference in freeze behaviour (n = 10, mean ± SEM : 26.11 ± 1.12 min, 13.37 ± 1.44 min, Tukey's post hoc, p < 0.001), Hide and Low activity behaviour (n = 10, mean ± SEM: 20.75 ± 2.08 min, 11.35 ± 1.22 min, 9.89 ± 1.5 min and 18.06 ± 1.08 min, respectively, Tukey's post hoc, p < 0.001) and Active behaviour (Inspection: n = 10, mean ± SEM: 23.88 ± 1.96 min and 9.05 ± 0.55 min: Move away: n = 10, mean ± SEM:11.34 ± 1.23 min and 28.01 ±1.53 min: Skitter: n = 10, mean ± SEM; 8.03 ± 0.96 min and 20.38 ± 2.68 min, respectively, Tukey's post hoc, p < 0.001) between captive reared and wild caught population (Fig. 5).

Table 3. Summary of the ANOVA examining the exploratory, feeding and anti-predatory behaviours of wild caught and captive reared *T. putitota*.

Exploratory behaviour					
Source of variation	**df**	**SS**	**Ms**	**F**	**p**
Population (wild and captive)	1	0.0311	0.0311	5.00E-04	0.9815
Behaviour (exploratory)	2	982.9614	491.4807	8.5303	<0.001
Interaction (population: behaviour)	2	9484.421	4742.211	82.3073	<0.001
Residuals	54	3111.258	57.6159	-	-
Feeding behaviour					
Population (wild and captive)	1	24855.42	24855.42	360.6753	<0.001
Behaviour (feeding on live prey)	2	7744.112	3872.056	56.1871	<0.001
Interaction (population:behaviour)	2	2889.454	1444.727	20.9643	<0.001
Residuals	54	3721.332	68.9136	-	-
Anti-predatory behaviour					
Population (wild and captive)	1	2.5037	2.5037	0.1425	0.7065
Behaviour (anti-predatory)	5	731.4963	146.2993	8.3276	<0.001
Interaction (population: behaviour)	5	4877.625	975.5249	55.5284	<0.001
Residuals	108	1897.347	17.568	-	-

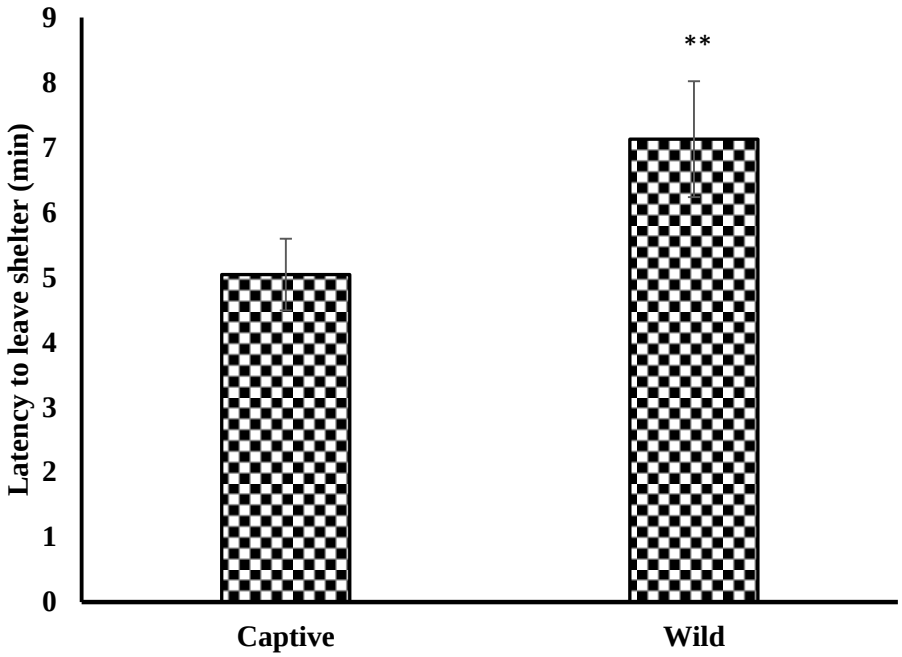


Fig. 1. Comparison of latency to leave the shelter (min) in captive reared and wild caught mahseer. Data are mean ± SEM (n=10). Bar with asterisks differ significantly ($p<0.05$). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ns = non-significant

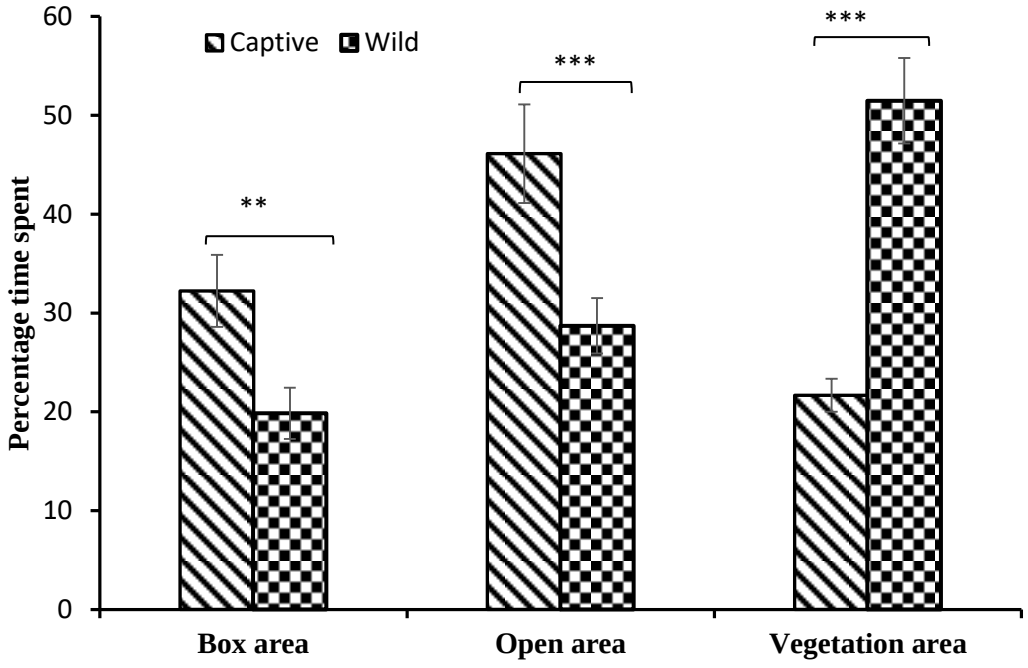


Fig. 2. Comparison of percentage time spent by captive reared and wild caught fish in a box, open and vegetation area. Data are mean ± SEM (n=10). Bar with asterisks differ significantly ($p<0.05$). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ns = non-significant

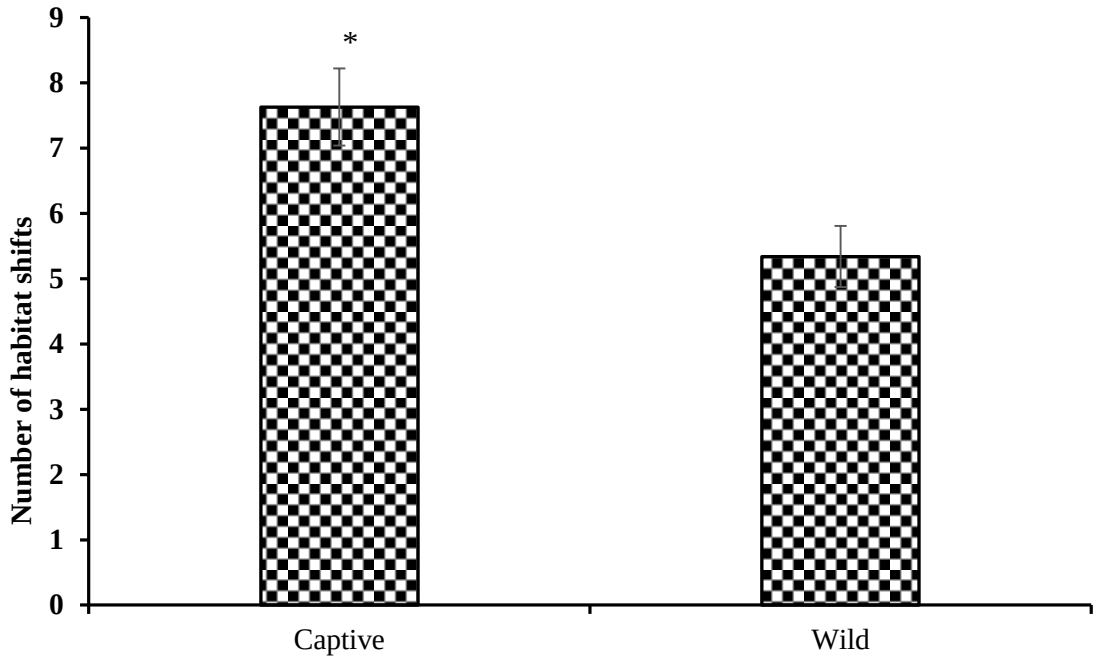


Fig. 3. Comparison of number of habitat shifts of captive reared and wild caught mahseer. Data are mean ± SEM (n=10) Bar with asterisks differ significantly ($p<0.05$). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ns = non-significant

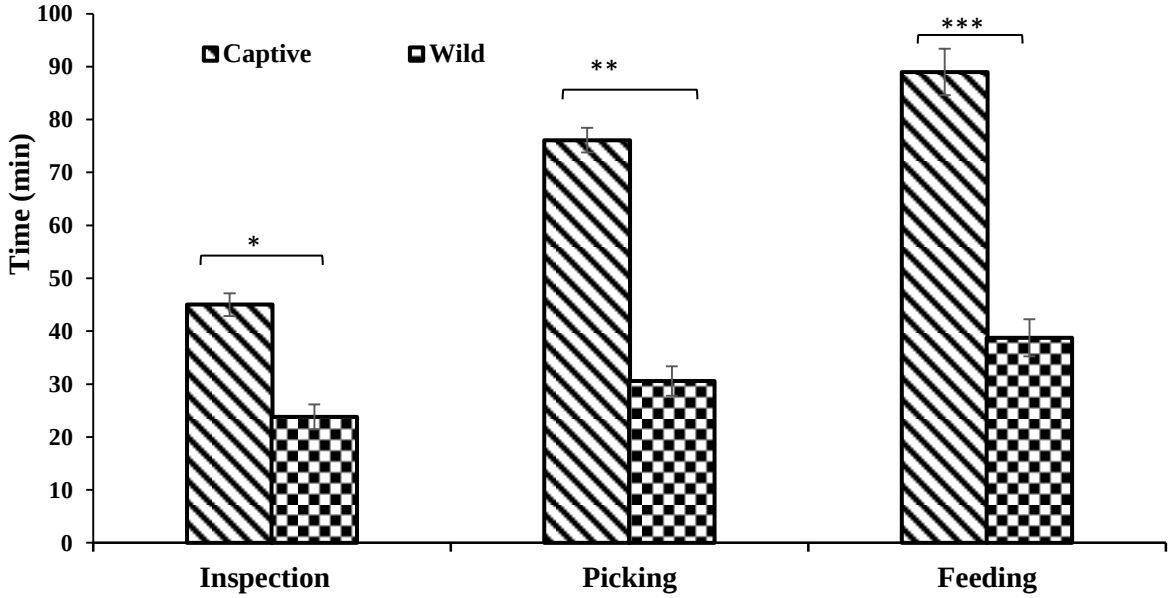


Fig. 4. Comparison of time spent in performing each behaviour, inspection, picking and feeding of live prey by captive reared and wild caught mahseer. Data are mean ± SEM (n=10). Bar with asterisks differ significantly ($p <0.05$). *$p < 0.05$; **$p < 0.01$; ***$p< 0.001$; ns= non-significant

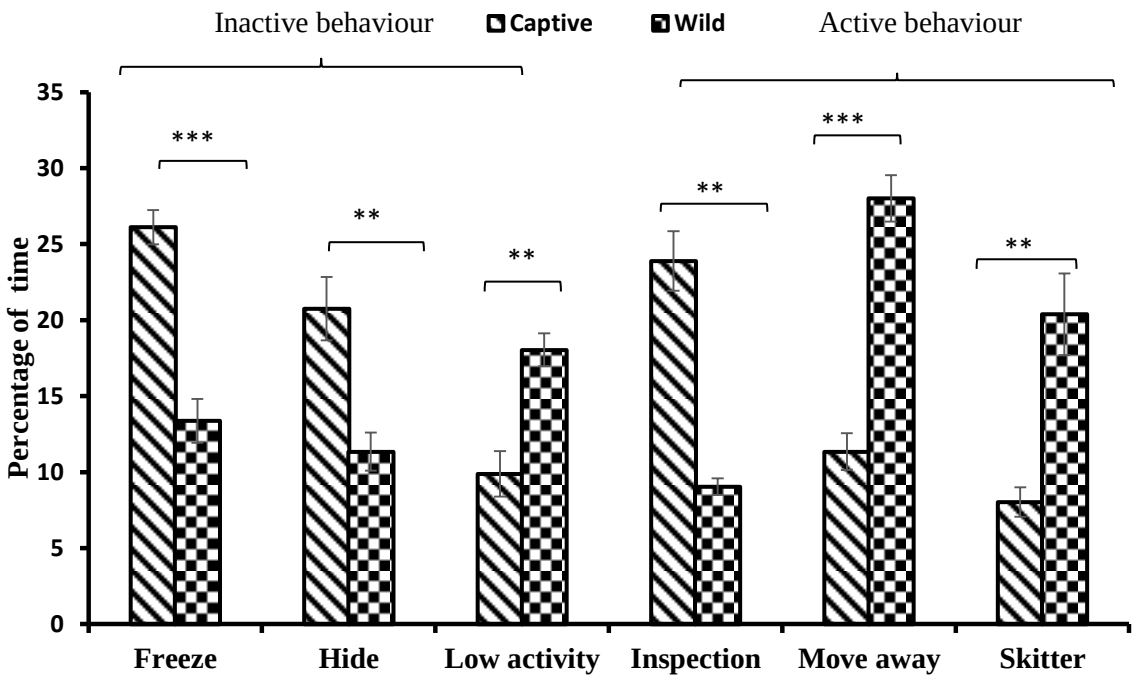


Fig. 5. Comparison of Percentage time spent, performing each active (freeze, hide, low activity) and inactive behaviour (inspection, move away, skitter) by two different population captive eared and wild caught mahseer. Data are mean ± SEM (n=10). Bar with asterisks differ significantly ($p<0.05$). *$p< 0.05$; **$p< 0.01$; ***$p< 0.001$; ns= non significant

4. Discussion

In the current study, the behaviour of mahseer collected from wild and captive rearing environment was evaluated. We applied different behavioural tests to compare the boldness, exploratory, predatory and anti-predatory behaviour and observed prominent differences in both populations. Captive rearing environment permits the expression of behaviour patterns quite different to be observed in wild caught fish. Previous observations have revealed that early experiences as well as rearing environments, shaping the innate behaviours like foraging, reproduction and the response to predators (Huntingford 2004; Braithwaite and Salvane, 2005; De Azevedo and Young, 2006; Kelley et al., 2006; Martin et al., 2015). We also detected disparity in the selected behaviours of the fish in all behavioural tests (Table 3).

The obvious differences in the behaviour pattern of both wild caught and captive reared mahseer could be the effect of variable experiences and rearing environments. It appears that captive environments permit the expression of bolder and more exploratory behaviour in hatchery reared fish. Boldness is a fearless behaviour like more rapid emergence from the shelter, and more frequent inspections of predator (Kelley et al., 2006). It is, generally, associated with fitness-related traits like aggression (Johnson et al., 2014), foraging and exploration (Wilson and Stevens, 2005) antipredator behaviour (Brown et al., 2007a; Lopez et al., 2005) mate selection (Godin and Dugatkin, 1996) and learning (Dugatkin and Alfieri, 2003). Captive rearing environment increases the boldness in fish (Sundström et al. 2004; Huntingford, 2004; Kelley et al., 2006), which could be due to high density at hatcheries as the animals need to compete for resources. Boldness, although at some level will be adaptive (Sih et al., 2004) but not always advantageous. The more curious and risk sensitive behaviours of bold animals as observed in captive reared fish, increase their conspicuousness and exposed them to potential predators (Kelley et al., 2006). Several studies have demonstrated the expression of boldness and low post release survivorship of hatchery reared fish in the natural environment (Huntingford, 2004; Johnsson et al., 2014). The tendency to take risks (e.g. Inspection of predators), be exploratory and spend more time in risky areas could explain the increase mortality of hatchery reared fish in wild (Jonsson, 1997).

Although , our observations of mahseer are similar to the earlier observations, as hatchery reared mahseer is bolder and more risk sensitive than wild caught fish (Fig. 1). However, the overall level of boldness in our captive-reared mahseer and wild counterpart was not as high as reported by other scientists for different fish species e.g., Atlantic cod *(Gadus morhua)* (Braithwaite and Salvanes, 2005), rainbow trout (*Oncorhynchus mykiss)* (Sneddon, 2003) and spotted skiffa (*Skiffia multipunctata)* (Kelley et al., 2006). It may be due to the fact that in Pakistan mahseer are reared in semi-intensive culture system where stocking densities are not as high as required for inducing high levels of boldness (Blanchet et al., 2006).

Environmental awareness is prerequisite for the survival of animals, while exploratory behaviour permit animals to collect information from surrounding environment more efficiently and rapidly (Braithwaite and Salvanes, 2005). Exploratory behaviour serve as an indicator of an animal adaptation to a novel environment and is under the influence of many factors like rearing environment, genetics, sex and maternal factor (Burns et al., 2016), body size (Brown et al., 2007a), brain size (Kotrschal et al., 2014), food availability and maternal effects (Burns, 2016). The captive reared fish in our study, spent more time in the open area and start box than wild caught fish (Fig. 2) . However, we cannot conclude that captive-reared fish are more exploratory than wild ones. Although, according to some scientist, fish spent more time in open area are more active (bold) and exploratory (Sneddon, 2003; Frost et al., 2007), but in the present study it appears that captive-reared fish is somehow reluctant to explore the area of vegetation, due to the previous rearing exposure and experience. The results of habitat shift clearly differentiate exploratory behaviour of captive-reared and wild caught mahseer (Fig. 3). Camacho-Cervantes (2015) studied exploratory behavioural on guppies and concluded that guppies engaged more suddenly in exploratory behaviour in the presence of vegetation. Kotrschal et al. (2014) concluded that large brain size guppies were more exploratory comparative to smaller brain guppies. These findings are similar to our current observations as wild environments contained more vegetation, than in captivity. Moreover, the fact that wild mahseer shows more exploratory behaviour, may be due to previous experiences. However, our findings are somehow different to Millot et al. (2009), who reported similar swimming activity and spatial exploration behaviour in both wild and domesticated sea bass strains (*Dicentrarchus labrax*). The

variations in results could be due to main difference in the generation time of fish used. We compared the wild caught fish from the fourth generation captive reared while Millot et al. (2009), compared the domesticated sea bass with hatchery reared counterpart produced from wild caught brooders.

Environmental variability can have positive effects on the attraction and consumption of live prey (Braithwaite and Salvanes, 2005). Earlier studies on salmonids suggest reduces foraging success of hatchery reared fish on live prey (Brännäs and Johnsson, 2008; Huntingford, 2004) and expression of boldness and risk sensitive behaviour in fish (Sundström et al., 2004; Kelly et al., 2006). Hatchery reared fish are often slow to adopt or switch to new prey items (Olla et al., 1998) and never reach the feeding efficiency comparable to wild fish, e.g., wild caught brown trout consumed 75% more live prey than hatchery reared fish and even in isolation, it utilized the prey more efficiently (Sundström and Johnsson, 2001). Consistent with other observations, wild mahseer in the present study also ate more, attacked quickly and consumed novel prey more efficiently than hatchery reared fish (Fig. 4). The divergence in feeding habits may be due to previous experience of wild fish to prey as earlier exposure and repeated experiences can improve the ability of fish to recognize, attack and ingest the prey (Warburton, 2003; Brown et al., 2003). Hence, reduced feeding ability of hatchery reared mahseer may be due to their lack of previous experience with live prey.

Many Intrinsic and extrinsic factors including hunger or gut fullness, isolation stress and threat plays important role in motivation of feeding (Hughes, 2013). For instance hunger reduces prey-handling time in fifteen-spined sticklebacks (*Spinachia spinachia*) (Hughes, 2013). However, in the present study, both wild caught and hatchery reared populations were under similar experimental conditions and on the same daily ration prior to the study. Thus, we assume that both hatchery reared and wild mahseer fish were similarly motivated and difference in behaviour is mainly due to their previous experiences and rearing environments.

Hatchery rearing environment lacks many of the natural aspects of a wild environment, most importantly predators, and as a result hatchery-reared fish cannot develop the acquired predator recognition and avoidance behaviours, important to

survive in the wild environment (Brown and Day, 2002). Previous literature has suggested that increased artificial culture time and selection for fast growth in aquaculture practices has resulted in dramatic anti-predatory behaviour differences between hatchery reared and wild caught individuals (Huntingford, 2004). Berejikian (1995) found that during live predator encounters, wild steelhead trout fry survived significantly better than their hatchery-reared (predator naïve) counterparts. Similarly, wild juvenile coho salmon (*Oncorhynchus kisutch*) consistently survived in greater ratio and for longer time periods when exposed to a live lingcod (*Ophiodon elongates*) than did hatchery-reared coho salmon (Olla and Davis, 1989). In our study, we compared the behaviour of hatchery reared and wild-caught mahseer exposed to a live predator and observed differences in all anti-predatory trials or in the time taken for the mahseer to inspect the predator (Fig. 5). Wild caught fish tended to show more active behaviour than captive reared fish in response to a predator. The result is in agreement with Oliver et al. (2008) who found that in the presence of a threat, lobsters raised without predators in the experimental tank showed reduced activity than those reared together with predators. The investigatory trips of both wild caught and captive reared mahseer towards the predator also showed a significant difference and support the view that captive reared fish always sooner launching investigatory trips as compared to wild counterpart (Sundström et al., 2004; Petersson and Järvi, 2006). Moreover, Kelley and Magurran (2003) observed more closer approaches of laboratory reared fish towards the model predator and suggested that such risk taking behaviour make captive-reared fish more prone to predation when subsequently released into the wild.

5. Conclusion

The present study indicated the differences in behavioural patterns (boldness, exploratory and anti-predatory behaviours) of wild caught and captive reared populations of mahseer and confirms the role of rearing environment in shaping the behaviour of fish. For improving the results of restocking program, the outcomes of this study suggest a modification of the hatchery rearing environment in such a way that could produce fish, with life skills comparable to their wild counterpart.

Chapter # 3

Effects of Early Rearing enrichment on Physiological Stress Response of Mahseer (*Tor Putitora*)

ABSTRACT

Enriching rearing environment is the strategy suggested for improving the post release survivorship of captive reared animals. Enriched rearing environment can have positive effects on physiology, health and survival of fish. Here an attempt has been to evaluate the impact of early rearing enrichment on the hypothalamic–pituitary–interrenal axis (HPI-axis), blood glucose as well as brain dopaminergic and serotonergic systems of *Tor putitora*. Fifteen days old hachlings of *T. putitora* were reared up to advanced fry stage in barren, semi- natural and physically-enriched environments and compared them with regard to pre stress and post stress levels of whole body cortisol, blood glucose, whole brain serotonergic activity (5HIAA/5HT ratio), dopaminergic activity (DOPAC/DA and HVA /DA ratios) and Norepinephrine (NE) levels. Significantly low basal whole body cortisol, glucose and brain NE levels were observed in a physically enriched group of fish as compared to other groups. However, after acute stress all rearing groups showed elevated levels of cortisol, blood glucose, brain 5HIAA/5HT, DOPAC/DA and HVA /DA ratios and NE levels but the magnitude of response was different among different rearing groups. The barren reared group showed higher magnitude of response as compared to semi natural and physically-enriched groups. Similarly, the recovery rate of whole-body cortisol, blood glucose and whole-brain monoamines were long lasting in barren reared mahseer. We illustrate that increased structural complexity (physical enrichment) during early rearing significantly modulate various physiological and stress coping mechanisms of mahseer

1. Introduction

Golden mahseer is the National fish of Pakistan that is widely distributed in foothills of Himalayan region and sub-continent (Chatta and Ayub, 2010; Bakawale and Kanhere, 2013). Earlier studies indicated the abundance of *Tor* species in most of the rivers of Pakistan (Mirza and Khan, 1994; Mirza et al., 1994; Zafar et al., 2001), but recent studies reported the decline population in most parts of the country (Chatta and Ayub, 2010) may be due to deforestation, pollution, overexploitation, damming and habitat destruction by initiation of hydro-power projects (Lakra et al., 2010; Nautiyal, 2011, 2014; Pandit and Grumbine, 2012; Khajuria et al., 2013; Gupta et al., 2014, 2015; Sharma et al., 2015).

By realizing the natural threat to this species, Pakistan has initiated artificial propagation and restocking programs for this species. The major aims of these programs are to rehabilitate and conserve this species. Since 2001, every year, thousands of seeds have been produced, reared in hatcheries and released into wild under restocking programs. However, in spite of extensive stock enhancement strategies; the population of this species is continually declining. It seems that like other fish species, captive hatchery reared mahseer do not survive after release in wild (Jonsson and Jonsson, 2006; Kallio-Nyberg et al., 2011). It has long been recognized that hatchery-reared fish release does not always bring remarkable improvements in fish stocks (Larscheid, 1995; Blaxter, 2000; Hutchison et al., 2006).

Poor post release survivorship of hatchery-reared fish can be attributed to a multiple factors, including stocking density, rearing environment (habitat), food availability, predator, exposure to disease organism, human interference (Huntingford, 2004). Hatchery reared fish develop trait would be detrimental in wild (Latremouille, 2003; Huntingford, 2004; Ashley, 2007). In hatcheries, fish are under the planned or un-intentional selection pressure, thus traits which are more suitable for captivity compared to the wild are developed (Frankhamet al.,1986; Huntingford, 2004; Christie et al., 2012). In fish reared for conservational stocking, behaviour adapted to natural environments are critical for stocking effectiveness (Brown and Day, 2002; Salvanes and Braithwaite, 2006). Recent experiments on the fish show that the lack of stimuli variation in captive rearing conditions, influences the phenotype at many

different levels, ranging from physiology to behaviour (Huntingford, 2004; Brännäs and Johnsson, 2008; Brockmark and Johnsson, 2010).

Several actions like life-skills training, environmental enrichment, social learning protocols, acclimatization at release have been suggested as solutions to reduce the behavioural deficiencies of hatchery reared fish (Brown and Day, 2002). It is suggested that enriched captive environments can promote shelter seeking abilities (Salvanes and Braithwaite, 2005; Roberts et al., 2011), behavioural flexibility (Braithwaite and Salvanes, 2005); foraging abilities (Brown et al., 2003; Strand et al., 2010; Rodewald et al., 2011), reduced anxiety and depression and enhanced learning/memory (Brenes et al., 2008; McQuaid et al., 2012; du Toit et al., 2012). However, barren hatchery rearing environment with high density alters their natural behaviours, e.g., social interactions, shoaling, shelter seeking and territorial behaviours (Barton and Iwama, 1991; Salvanes and Braithwaite, 2005; Roberts et al., 2011; Batzinaet al., 2014). Generally, environmental enrichment (social and physical) enhances the performance and improves the welfare of animals held in captivity. It not only influences the behavioural traits, but also fulfil the psychological needs of animals in captivity (Shepherdson et al., 1998).

In captivity fish are subjected to several environmental, social, and husbandry related stimuli that may have potentially noxious or stressful effects. Stress has been defined as a physiological cascade of events that occurs when an individual attempts to re-establish homeostasis in the face of a perceived threat (Schreck et al., 2001). Upon exposure to a stressors, fish undergo a series of behavioural, physiological, neuroendocrine and biochemical changes (Barton, 2002; Davis, 2006) in order to cope with any potential threat (Barton, 2002). Stress responses of fish can show variation within and between species, between strains, and even among individuals (Barton, 2002) and these differences are genetically based but influenced by individual experience (Heath et al., 1993; Overli et al., 2005). Individuals show variation to cope stress because stress responses are controlled by hormones (Huntingford et al., 2010; Koolhaas et al., 2007) having moderate to high degree of heritability (Overli et al., 2005). However, it is well documented that hatchery-reared and wild caught often differ in their physiological stress responses (Lepageet al., 2000) may be due to genetic environment interaction

The physiological responses of fish to stress may be of either an adaptive nature, allowing for homeostatic recovery, or a maladaptive nature, having adverse effects on survival, growth, immune response, reproductive capabilities, behaviour and general fitness (Schreck et al., 2001; Kelley et al., 2006; Magnhagen et al., 2008). The primary stress response in fish includes the rapid increase of circulating catecholamines and cortisol, while the secondary response includes changes in several hematological and biochemical parameters; the tertiary response involves alterations at the whole animal and population level (WendelaarBonga, 1997; Barton, 2002). In teleosts, catecholamines and corticosteroids are the respective main end-products of the two major pathways coordinating the stress response: the brain–sympathetic–chromaffin cells and the hypothalamus–pituitary–inter-renal cells (HPI) axes (Balment et al., 2006; Clark et al., 2011; Guesto et al., 2013; Biamonte et al., 2015).

In addition to HPI axis, brain monoaminergic neurotransmitters also involved in regulation of stress responses (Winberg et al., 2001; Bowman et al., 2002; Larson et al., 2003; Perreault et al., 2003; Lepage et al., 2005; Gesto et al., 2013). Like in other vertebrate fish also showed elevated level of brain serotonergic activity, as indicated by an increase level of brain 5-hydroxyindoleacetic acid (5-HIAA) and ratio of 5-HIAA to serotonin (5-hydroxytryptamine/ 5-HT) (Øverli et al.,1999, 2001; Winberg et al., 2001; Barton et al., 2008; Gesto et al., 2013).

Data derived from several studies on fish indicate the beneficial effects of environmental enrichment on metabolic demand (Millidine et al., 2006; Finstad et al.,2007), stress activity (5-HIAA/5-HT) (Batzina et al., 2014; Höglund et al., 2005), cognitive abilities (Brown et al., 2003; Brenes et al., 2008; Kotrschal and Taborsky, 2010; McQuaid et al., 2012; du Toit et al., 2012), behaviour (Salvanes and Braithwaite, 2005; Salvanes et al., 2007; Moberg et al., 2011; Roberts et al., 2011) and survival in the wild (Maynard et al., 1996).

We assumed that by enriching the early rearing environment at hatcheries, physiology of *T. putitora* with regard to stress response can be modulated and fish can be prepared to cope the challenges of common stressor present in nature. The major objective of the present study was to evaluate the effect of the early rearing enrichment on stress responses in term of HPI axis and activities of dopaminergic and

serotonergic systems of advanced fry of mahseer previously reared in different rearing environments. To test our assumptions, we devised three different rearing environments that differ in their levels of complexity and heterogeneity. Mahseer hatchlings were reared up to advanced fry stage in these three different rearing environments and then compared their stress response by measuring the recovery rate of plasma cortisol (invasive), water-borne cortisol (non-invasive), blood glucose and brain monoamines (ratios of 5HIAA/5HT, DOPAC/DA and HVA /DA) and NE level after acute physical stress.

2. Material and methods

2.1. Experimental Animals

Tor putitora 15 days, hatchlings (10-15mm; after semi-quiescent staged), about 25000 in number, were collected from a breeding tank (barren concrete circular tank) of Fish Hatchery Hattian Attock, Pakistan, and transported in oxygen filled plastic bags (36cm length × 24cm width; 10 L water) of the Fisheries and Aquaculture Research Station, Department of Animal Sciences, Faculty of Biological Sciences, Quaid-I-Azam University Islamabad, Pakistan.

2.2. Manipulation of rearing environment

To investigate the effect of early rearing environment on exploratory, predatory and anti-predatory behaviours, the hatchlings of mahseer (*Tor putitora*) were housed in three different rearing environments, 1) Barren: Rectangular fibreglass tanks (120 cm length × 60 cm width × 60 cm height) without any substrate (Clear bed), while plain contained only an aerator. 2) Physically enriched: Similar size fibreglass rectangular tanks (120 cm length × 60 cm width × 60 cm height) with 1 cm thick 1.0 to 1.5 cm diameter gravel bed, 4 plastic plants having 12 cm height, 2PVC pipes (10 cm length; 4 cm diameter and 5 cm length; 4 cm diameter and aerator. 3) Semi-natural: 250 m^2 earthen pond (20m × 12.5 m) with concrete walls, having natural food organisms (phytoplankton, zooplankton, diatoms, some protozoa etc.) but without common aquatic weeds or other substrate. Before stocking, the ponds were limed (calcite lime; CaCO3; CaCO3; 49.42 kg/100 m2, fertilized with organic (Animal manure; cow dung 8 kg/100 m^2/week) and inorganic (Nitrogen fertilizers; 0.5 kg/100 m2/week; phosphate fertilizers; 0.25 kg/100 m^2/week) fertilizer, thus to contain a sufficient amount of natural food organisms (A conventional technique in Pakistan for the rearing of mahseer). Replicate of three for barren and physically enriched and two for semi-natural environments were maintained and hatchlings were stocked at the rate of 200 hatchlings per tank and about 10,000 hatchlings per semi-natural pond. Initially, low water level about 0.6 m in each earthen pond and 200 L in fibreglass tanks was maintained, but after two months raised to 1 m and 350 L respectively. All experimental groups were supplied with water from a nearby

freshwater stream (Rumli freshwater stream). Hatchlings in barren and physically enriched groups were initially fed commercially available prepared powder feed (Oryza organics fish feed, powder; 55% crude protein, 12% lipid fats, crude fiber 2% and 10% moisture) after every second hour, then gradually changed from powder feed (first two months)to crumbled (after two months) and then sinking pellets (last one month) (Oryza organics fish feed, size 2 mm; 45% crude protein, 14% fats, 2% crude fiber and 10% moisture), at the rate of 4% body weight twice per day. In the ponds sufficient natural food organisms were maintained with the aid of fertilizer. Additionally, some prepared feed was also provided daily. Pond fertility was checked every fortnight with Sacchi disk and accordingly taken the step (Almazan and Boyd, 1978). The fish was reared under these conditions for 4 months (March, 2015 to June, 2015). During rearing, optimum oxygen levels in all tanks were maintained by using aerators, pH and temperature were noted daily. Moreover, to avoid strong response towards the handling and netting stress, all fish were inspected daily during the experimental time period. After completion of rearing period, fish from each experimental group were randomly selected for physiological stress response

2.3 Stress assay

2.3.1 Stress assay procedure for mahseer from barren, semi natural and physically enriched environment

For evaluating the impact of rearing environment on the physiological stress response of fish, *T. putitora* previously reared in barren, semi-natural and physically enriched environment re-housed at a stocking density of 10.06 ± 0.05 gL^{-1} in eighteen, 25L volume glass aquaria of the same size (40L×25W×25H cm), six aquaria for each environment. Barren and physically enriched groups of fish were almost similar in size (body mass, 2.14 ± 0.04 and 2.17 ± 0.05g respectively) as compared to a semi natural pond (body mass, 2.26 ± 0.08g). Here again size difference was controlled by maintaining the stocking density of both populations instead of the number of fish in aquaria. The experiment was conducted in replicate of three, with slight variation in number of fish in aquaria. Both, barren and physically enriched reared fish similar in number in each aquaria (n=19) while

semi-natural environment reared fish showed variation (19, 19, 19, 19, 18 and 18) . The six aquaria of each group were split into half for control and other for treatment i.e. exposure to stress. All aquaria had permanent volume marks for maintaining water volume and were well equipped with aerators and water heater, to maintain a constant temperature of 22.5˚C. They were kept under flow through the system (10.02 ± 0.03 $mLmin^{-1}$). Water to each aquaria was supplied from the water tank (100 gallon) having dechlorinated water and set at a temperature (22.5 ± 0.5˚C) similar to experimental aquaria. Water from the tank was supplied through the main pipeline having an outlet equipped with water flow regulator at the top of each aquaria, while the outflow of water from aquaria was controlled by Tygon® tubing with regulator. During the experiment, the pH ranged from 6.9 to 7.2, DO was near saturation (>6.4 mg L^{-1}), ammonia was less than 0.20 ppm, and salinity was 0.0 ppm. Fish were fed once daily at 09:00 am with floating feed (Oryza organics fish feed, pelleted; 55% crude protein, 12% lipid fats, crude fibre 2% and 10% moisture). Every day uneaten feed and faces were removed by siphoning and about 20% water in each aquarium was also maintained by addition of water. The experiment consists of two treatments; control, i.e. unstressed fish and treated, i.e. exposed to acute stress. Treated groups were stressed by following the same handling stress protocol as used for evaluating the stress response of wild and captive populations of mahseer i.e. 5 min chasing with hand net and 2 min confinement at the corner of the aquarium.

2.3.2 Water sampling

Water samples were collected by adopting the same procedure as described in Chapter # 1 Page # 24-26.

2.3.3 Extraction procedure of Water-borne Cortisol

Extraction of free cortisol from pre-stress and post stress samples were also done by adopting previously mentioned methodology (Chapter # 1, Page # 26)

2.4 Experimental protocols for blood glucose, Whole body cortisol and Brain monoamines

2. 4.1 Distribution of fish and stress assay procedure

Before performing the stress assay procedure, a total of 360 advanced fry of mahseer from three different rearing environments were distributed (12 fish per aquarium; stocking density, 5 g/L) among 30 same size (40 L×25W×25H cm; 25 L water) experimental aquaria (differ in structural enrichment) under flow through systems (10 ± 0.05 ml min^{-1}). In order to mimic their previous rearing environment , i) Fish (n=120, body mass, 2.14 ± 0.04) previously reared in the barren environment were distributed in ten aquaria without any substrate (Clear bed; two aerators).ii) Fish (n=120,body mass, 2.26±0.08) previously reared in semi-natural environment were distributed in ten aquaria having water from semi-natural ponds (turbid water; phytoplankton, zooplankton, protozoa etc.), while iii) Fish (n=120, body mass, 2.17±0.05) previously reared in physically-enriched environment were distributed in ten physically-enriched aquaria with gravel bed, plastic plants, PVC pipes and two aerators). After the distribution, the fish were remained in their respective environment without any disturbance for a period of one week under same laboratory condition (12:12hr, light: dark; temperature, 22.5°C; DO>6.4; mgL^{-1} and ammonia < 0.20 mgL^{-1}). After one week, fish in one experimental aquarium of each group were anaesthetized by adding (MS-222; 50mgL^{-1}) to the water and 6 fish from each aquarium were collected for obtaining a basal level of whole body cortisol (invasive method) and another six for blood glucose and brain monoamines basal levels. The fish collected for blood glucose and brain monoamines assay were quickly picked and approximately 20 µL of blood was collected from the caudal vasculature, by sharp cutting of fish, tail and dissected out immediately (within 3 min) and their brain was deep frozen in liquid nitrogen and stored at -80°C for further analysis of monoamines. However, fish for whole body cortisol analysis also deep frozen in liquid nitrogen and stored at -20°C.

In order to find out the comparison of the stress response of fish originated from different rearing environment, fish in remaining aquaria were exposed to acute handling stress by following the procedure previously adopted by Guesto et al. (2013) for rainbow trout. Briefly, fish were chased 5 min with hand net and 2 min confinement at the corner of aquaria and samples were collected by using the same technique at 0.5, 0.75, 1, 2, 4, 6, 8, 24 and 48hr after acute stress. Samples from each environmental group were collected as described previously for wild and captive

reared fish, i.e. every time from new aquarium requisite for a particular time period (1 aquarium /time period / environment).

2.4.2 Blood Glucose Level: stress assay procedure

The blood glucose level was analysed with the help of digital glucometer ACCU-CHEK®Softclix (blood glucose meter). A drop of fresh blood was placed on the glucometer strip. The Glucometer showed the result on screen in mg/dL^{-1}. The unit of glucose converted from mg/dL to mmole/L by dividing the value of glucose by 18.018.

2.4.3 Whole body Cortisol

Fish were small in size, therefore not possible to collect sufficient blood for plasma cortisol. Cortisol was extracted from whole-body by using the method reported by (Zuberi et al., 2014), and previously used by Sink et al. (2007) for golden shiners and Yeh et al. (2013) for zebra fish larvae. Whole frozen individual advanced fry of mahseer was thawed, removed excess water by using paper towel, weighed and placed on a glass plate. The individual was sliced into small pieces and crushed with the help of sharp blades. The paste was transferred into the glass tube with 500µL phosphate buffer saline (PBS) and homogenized at 10000 rpm for 60-70 sec by using electric homogenizer (Model No. AHS 200). During homogenization, sample tube was placed in a beaker filled with crushed ice. Probe of homogenizer was washed into the sample tube with an additional 500µL aliquot of PBS. Each sample was extracted three times with 8 volumes of ethyl acetate. During extraction, tubes were vigorously vortex with ethyl acetate for 60s and centrifuged at 3000 rpm for 10 min at 4°C to separate the aqueous and Ethyl acetate layers. The bottom layer (aqueous homogenate) was frozen at -80 °C for 15 min and the top ethyl acetate containing extracted steroid hormones was collected into a new glass test tube (16×100 mm). The glass tube was set in a water bath set at 45°C and samples were dried under a gentle stream of nitrogen gas. The dried lipid extracts were stored at –20 °C until conducting ELISA for cortisol.

2.4.4 Water-borne and whole body cortisol estimation

For estimation of cortisol in water and whole body sample, the same kit as previously mentioned (Chaper #1 and Page # 28) was used. The cortisol kit was also validated for whole body cortisol concentrations and observed that the slope of the curve created by kit standards matched with curve obtained by serial dilutions of samples (0, 20, 40, 60 and 80 %) (slopes, whole body cortisol: slope = 0.932, r^2 = 0.998, P = 0.96) reflecting the positive linear relationship. The intra-assay (for precision of kit) and inter assay (reproducibility) coefficient of variation was calculated and found to be 9.7 and 12.5 % respectively.

Furthermore, cortisol extraction efficiency from whole body was investigated by adding predicted concentrations of cortisol (20, 40 , 60 and 80 ng g-1) in charcoal stripped whole body homogenate. The cortisol was extracted and estimated by following the same procedure as used for samples. The % recovery of cortisol was greater than 96.8%. Absorbence at 450 nm was read in ELISAplate reader.All cortisol values are reported as, ngL^{-1} for water borne cortisol and ngg-1 for whole body cortisol.

2.5 Monoamines assay procedure

The analysis was done by adopting the same extraction protocol and estimation procedures as mentioned previously (Chapter # 1, Page # 29)

2.7 Statistical analysis

All results are mentioned as mean (± SEM). Statistical analysis was carried out by using lme4 (Bates et al., 2014) and easyanova (Arnhold, 2013) package of R 3.2.5 (R Development Core Team, 2016). The assumption of normality, homogeneity of variances and additivity of the model were checked by *Shapiro-Wilks*, *Levene'*sand *Tukey1-dFTest* respectively. The effects of rearing environment on blood glucose, whole body cortisol and brain monoamines were analysed by using ANOVA with double factorial in a complete randomized design followed by post hoc *Tukey's HSD.* Results considered statistically significant, when value was less than 0.05 ($P<0.05$).

Correlations between whole body cortisol and brain monoaminergic activity and whole body cortisol with glucose were tested by the Pearson's correlation test.

3. RESULTS

3.1.Water-borne cortisol

Basal levels of concentrations of advanced fry of mahseer reared in three different environments (barren, semi natural and physically enriched) were statistically similar (Tukey's post hoc: $p>0.05$, Table 2, Fig 1), while after induced acute stress, increase in all rearing groups. The repeated way analysis indicated that concentration changed over time (two way ANOVA $F_{1,\ 220} = 2153.858$, $p<0.001$, Table 1), while significant interaction between rearing groups and treatment (two way ANOVA $F_{20\ 330} = 4.122$, $p<0.001$, Table 1) indicated the manner in which rearing groups varied in physiological stress response. However, concentration of cortisol in control of all treated groups did not show significant fluctuation over time ($P>0.05$). The significant three-way interaction between variables (two way ANOVA, $F_{20,\ 330} = 4.888$, $p<0.001$Table 3) suggest the variation in patterns displayed by three rearing groups in response to acute stress over time. After stress all rearing groups (barren, semi-natural and physically enriched) showed their peak level of water borne cortisol at 2 hrs after stress but the cortisol concentrations of barren and semi-natural reared fish were statistically similar ($P=0.11$) as compared to barren verses physically enriched and semi-natural versus physically enriched rearing groups ($P=0.001$ and $p=0.001$ respectively).

3.2. Whole Body Cortisol

The pre-stress whole-body cortisol level was significantly different in advanced fry of mahseer reared in physically-enriched environment compared to barren and semi-natural environments (Tukey's post hoc:$p=0.05$ and $p=0.004$ respectively; Table 4, Fig. 2), while no significant difference was observed in barren and semi-natural environments reared fish (Tukey's post hoc: $p=0.52$, Table 4). The significant interaction between rearing environment and treatment (two way ANOVA: $F_{2,\ 150} = 4.3879$, $p<0.01$, Table 3) indicated how the stress response of fish reared in

barren, semi-natural and physically enriched rearing environment varied. The whole body cortisol concentration changed after exposure to stress as compared to pre-stress (two way ANOVA: $F_{1,\ 150} = 272.905$, $p<0.001$; Table 3), also changed over time and showed variable concentration at most of the studied hours among barren, semi-natural and physically enriched environments reared fry (two way ANOVA: $F_{8,\ 150} = 240.6765$, $p<0.001$, Table 3). The significant interaction of rearing groups with treatment (two way ANOVA: $F_{2,\ 150} = 4.3879$, $p<0.001$) as well as with time (two way ANOVA: $F_{16,\ 150} = 4.2074$, $p<0.001$) indicated how different rearing groups differ in their stress response. Although all groups attained peak level after 0.75 min of stress, but cortisol concentration and the trend to attain peak values were significantly different (Table 4, Fig. 2). At 0.25 min semi-natural environment reared fish showed rapid increased and highest level of cortisol but afterward trend changed and at the 0.75 min highest level was observed in barren reared fish followed by semi-natural environment reared fish (n = 6, mean ± SE: 96.5 ± 4.50 and 85.65 ± 2.31 and 71.5 ± 2.59 ngg^{-1} fish respectively, Tukey's post hoc: p=0.001, Table 4, Fig 2). After word cortisol level showed decreasing trend, but physically enriched and semi-natural environment reared fish showed rapid recovery and at 6 hrs attained levels statistically comparable to their pre-stress levels as compared to barren reared fish, where it remained high even after 8 hrs. At 24hr post stress, whole body cortisol concentrations of all groups were statistically compared to their respective pre-stress values (Table 4, Fig 2).

3. 3. Blood Glucose

Before stress, blood glucose level of semi-natural environment reared fry was significantly higher than physically enriched environment reared fish (Tukey's post hoc: p=0.002; Table 6, Fig. 3). But statistically comparable to barren reared fish (Tukey's post hoc:p=0.92; Table 6, Fig 3). However, barren and physically enriched environment reared fish showed significant difference in pre-stress blood glucose level (Tukey's post hoc:p=0.008, Table 6, Fig 3). After treatment, i.e. exposure to acute stress, a significant difference between barren, semi-natural and physically enriched environment reared fish (two way ANOVA: $F_{1,\ 150} = 1678.765$, $p<0.001$, Table 5),changed in glucose level over time (two way ANOVA: $F_{8,\ 150} = 822.259$, $p<0.001$, Table 5) and interactions between different rearing groups of fish with time

(two way ANOVA: $F_{16, 150} = 15.612$, $p<0.001$) and treatment (two way ANOVA: $F_{2, 150} = 16.799$, $p<0.001$, Table 5) were observed. Like whole body cortisol, plasma glucose of all rearing groups also differs in magnitude at peak concentration, highest in barren followed by semi-natural and the lowest in a physically enriched environment reared fish . Although, all groups attained peak values at 2 hr, but semi-natural environment reared fish showed initial rapidly increased at 0.25 min as compared to steady increased in concentration, observed in barren and physically enriched environment reared fish. After stress, semi-natural and physically enriched environment reared fish recovered their pre-stress level earlier, i.e. at 24 hrs than barren reared fish.

3.4. Brain Monoamines

3.4.1 Serotonergic activity (5HIAA/5HT ratio)

The pre stress whole brain serotonergic activity (5HIAA/5HT ratio) of advanced fry of mahseer from three rearing environments, barren, semi-natural and physically-enriched environment were considerably similar (Tukey's post hoc: $p>0.05$, Table 8, Fig. 4). Exposure to stress showed changes in the 5HIAA/5HT brain ratio (n = 6, mean ± SEM: ANOVA: $F_{1, 150} = 107.383$, $p < 0.001$, Table 7) over time (n = 6, mean ± SE: ANOVA: $F_{8, 150} = 144{,}772$, $p < 0.001$) at different level in three rearing groups fish (n = 6, mean ± SEM: ANOVA: $F_{2, 150} = 48.8568$, $p < 0.001$). The significant interaction between time and rearing groups (ANOVA: $F_{16, 150} = 2.248$, $p < 0.001$, Table 7) suggesting that in different rearing group, 5HIAA/5HT ratio changed with time while non significant interactions between Treatment × rearing group (ANOVA: $F_{2, 150} = 2.073$, $p = 0.052$, Table 7) indicated that change in 5HIAA/5HT ratio occurred independent of the rearing environment. All rearing groups after stress attained their peak level at 0.75 min but differ in 5HIAA/5HT ratio. Both semi-natural and physically enriched environments reared fish had significantly low 5HIAA/5HT ratio compared with barren reared fish (Tukey's post hoc: $p=0.05$ and $p=0.001$, respectively, Table 8), while semi-natural and physically enriched environments reared fish had a similar ratio (Tukey's post hoc: $p=0.09$). After peak ratio, there was a steady and continuous decline in following time period. Moreover, if we look more closely, it appears that different rearing groups showed a somewhat

difference in the recovery period. Both groups of fish reared in semi-natural and physically enriched environments attained a comparable basal level ratio of 5HIAA/5HT earlier, i.e. at 4 hrs after stress as compared to barren reared fish which attained the level at 6 hrs (Table 8, Fig. 4). It was also observed that at 2 hr a clear difference in the ratio of 5HIAA/5HT was noted between the rearing groups (Tukey's post hoc: $p<0.01$) while at 4 hr semi-natural and physically-enriched environment reared did not show significant differences (Tukey's post hoc: $p=0.10$). However, at 6 and 8 hr only a difference was observed between barren and physically enriched tank (Tukey's post hoc: $p=0.02$ and $p=0.04$; respectively, Table 8), while at 24 and 48 hr of stress assay, there was no significant difference in different rearing groups with respect to 5HIAA/5HT ratio.

3.4.2 Dopaminergic activity index DOPAC/ DA ratio

Before stress, whole brain dopaminergic activity index (DOPAC/DA ratio) in an advanced fry of mahseer reared in three different rearing environments were almost similar in range (Tukey's post hoc: $p>0.05$, Fig. 5, Table 10), while after acute physical stress, it showed a change (ANOVA: $F_{1,\ 150} = 67.7654$, $p < 0.001$, Table 9) with time (ANOVA: $F_{8,\ 150} = 78.720$, $p < 0.001$, Table 9) in all rearing groups and attained peak level at 0.75 hr. The non significant interaction between time and rearing groups (ANOVA: $F_{16,\ 150} = 1.551$, $p > 0.056$ Table 9) as well as between treatment and rearing group (ANOVA: $F_{2,\ 150} = 2.9318$, $p > 0.05$ Table 9) indicated that although DOPAC/ DA ratio changed in all groups over time after treatment, but barren and semi-natural reared fish showed almost similar ratio at all time periods while semi-natural and physically enriched at most of the time period except at 0.25 and 0.5 hr. However, barren and physically enriched environment reared fish showed significant ($p < 0.05$) variation in the DOPAC/ DA ratio at most time periods except at 0.25, 24and 48hr. Both physically enriched and semi-natural environment reared fish recovered their pre-stress level earlier, i.e. at 4 hrs as compared to barren reared fish which achieved this level at 8 hrs (Table 10, Fig. 5).

3.4.3 Norepinephrine (NE)

No significant difference was observed in pre-stress brain nor-epinephrine (NE) level of barren and semi-natural environment reared fish(Tukey's post hoc; p=0.611, Table 12, Fig. 6), while physically-enriched environment reared fish showed a significant difference with barren and semi-natural environment reared counterparts (Tukey's post hoc; p=0.05 and p=0.02 respectively, Table 12). After exposure to acute stress, all rearing groups showed significant increases in NE level as compared to their pre-stress level (ANOVA: $F_{1,\ 150} = 885.972$, $p < 0.001$; Table 11), that change over time, peak at 0.75 hr and then decline gradually up to 48 hrs (ANOVA: $F_{8,\ 150} = 1188.614$, $p < 0.001$, Table 11). The significant interaction between variables indicates how the different rearing groups differ in physiological stress response, i.e. at most of time period showed significant difference in NE level. If we look more closely at the results, it appears that during recovery, physically enriched rearing group attained comparable pre-stress level of NE earlier followed by semi-natural reared group, while barren reared fish had taken more time to attain normal levels (Table 12, Fig. 6).

3.4.4HVA/DA ratio

Pre-stress brain HVA/DA ratios of advanced fry of mahseer reared in three different rearing environments were statistically similar (Tukey's post hoc: $p > 0.05$, Table 14, Fig. 7). After exposure to acute stress, there was significant changed in ratio (two way ANOVA: $F_{1,\ 150} = 23.99$, $p < 0.001$), at different time period (two way ANOVA: $F_{8,\ 150} = 39.127$, $p < 0.001$, Table 13). There was no interaction between rearing groups and treatment (two way ANOVA: $F_{2,\ 150} = 1.9406$, $p = 0.14$, Table 13) as well as between rearing groups and time (two way ANOVA: $F_{16,\ 150} = 1.3616$, $p = 0.16$, Table 13). The barren and semi-natural environment reared fish did not show any significant difference (Tukey's post hoc: $p > 0.05$, Table 14) in HVA/DA ratios at different time periods while between semi-natural and physically enriched environmental groups, significant difference was appeared at 0.25 and 0.5 hrs. However, the difference in HVA/DA ratios between barren and physically enriched rearing groups was more prominent, as former group had a significantly higher ratio at 0.25, 0.5. 0.75 and 2 hrs. Both semi-natural and physically enriched environment groups recovered their pre stress HVA/DA ratio earlier, i.e. at 4 hrs as compared barren reared group which attained this at 6 hr after stress (Table 14, Fig. 7).

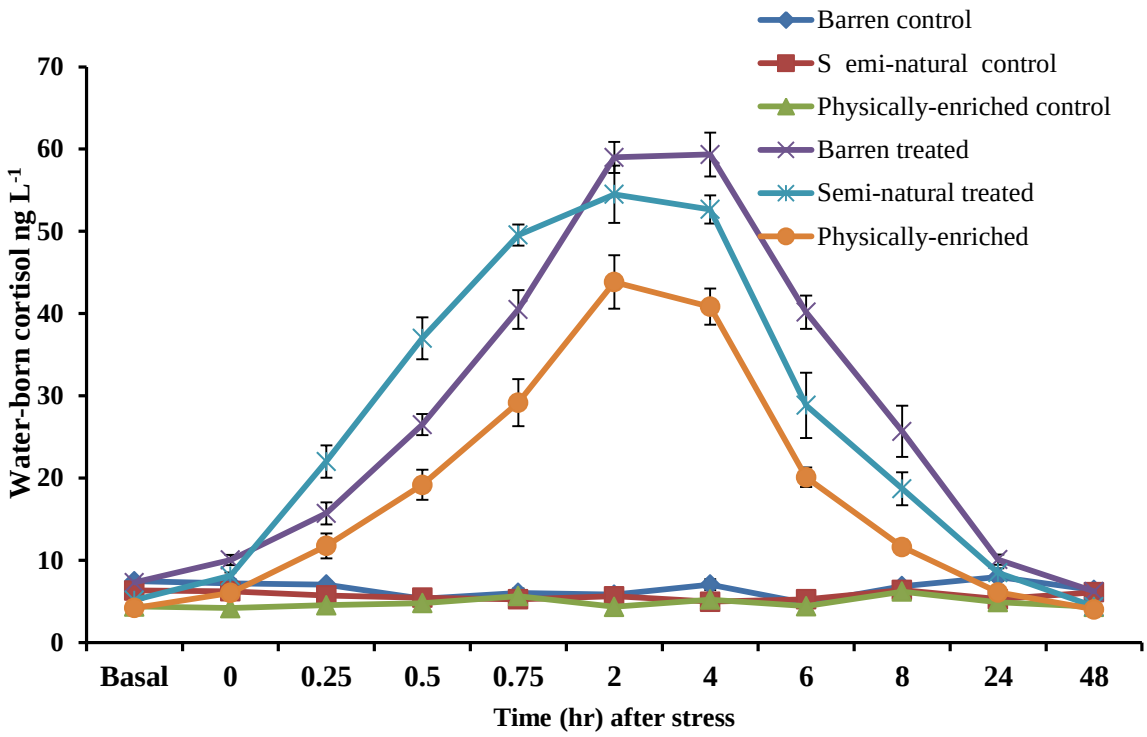


Fig. 1. Mean (± SEM) water-borne cortisol (ngL^{-1}) of barren, semi-natural and physically enriched environment reared *T. putitora* (n = 6) subjected to acute physical stress and sampled at various time intervals. Basal mean pre-stress level.

Table 1. Summary of the ANOVA examining water-borne cortisol in barren, semi-natural and physically-enriched environment reared *T. putitora* during control and acute physical stress treatments.

Source of variation	**Df**	**SS**	**MS**	**F**	**p**
Rearing Groups	2	2435	1217	78.879	< 0.00
Treatment	1	33241	33241	2153.858	< 0.001
Time	10	27578	2758	178.69	< 0.001
Treatment : Rearing Groups	2	1224	612	39.639	0.001
Time : Rearing Groups	20	1272	64	4.122	0.001
Time: Treatment	10	28596	2860	185.288	< 0.001
Time: Treatment: Rearing Groups	20	1509	75	4.888	0.001
Residuals	330	5093	15		

Table 2. Mean (±SEM) water-borne cortisol (ngL-1) from barren, semi-natural and physically-enriched environment reared *T. putitora* (n=6) subjected to acute physical stress and sampled at various time intervals. Basal mean pre-stress level.

Time (hr)	Rearing groups						Statistical Comparisons								
	Control			Treated			Between Control group			Between treated groups			Control vs Treated		
	Barren	Semi-natural	Physically-enriched	Barren	Semi-natural	Physically-enriched	Be-Se	Be-Pe	Se-Pe	Be-Se	Be-Pe	Se-Pe	Be-Be	Se-Se	Pe-Pe
Basal	7.44±0.28[a]	6.31±0.55[a]	4.36±0.48[a]	7.28±0.55[e]	5.14±0.49[e]	4.19±0.23[e]	0.83	0.36	0.66	0.61	0.36	0.90	0.94	0.60	0.94
0	7.1±0.36[a]	6.21±0.33[a]	4.18±0.23[a]	10.19±0.63[de]	8.29±0.41[e]	6.01±0.53[de]	0.89	0.64	0.64	0.67	0.15	0.57	0.09	0.36	0.41
0.25	7.02±0.44[a]	5.71±0.30[a]	4.51±0.35[a]	15.69±1.35[d]	22±1.95[cd]	11.75±1.52[d]	0.83	0.51	0.85	0.01	0.19	0.001	0.001	0.001	0.001
0.5	5.31±0.33[a]	5.45±0.54[a]	4.78±0.62[a]	26.48±1.28[c]	37±2.55[b]	19.16±1.82[c]	0.99	0.95	0.97	0.001	0.001	0.003	0.001	0.001	0.001
0.75	6.025±0.5[a]	5.25±0.66[a]	5.63±0.73[a]	40.5±2.35[b]	49.55±1.29[a]	29.16±2.85[b]	0.93	0.81	0.96	0.001	0.001	0.001	0.001	0.001	0.001
2	5.81±0.72[a]	5.61±0.38[a]	4.33±0.23[a]	58.98±1.89[a]	54.5±3.47[a]	43.83±3.26[a]	0.99	0.79	0.83	0.11	0.001	0.001	0.001	0.001	0.001
4	7.03±0.67[a]	4.95±0.54[a]	5.2±0.41[a]	59.33±2.67[a]	52.66±1.71[a]	40.83±2.19[a]	0.69	0.62	0.99	0.009	0.001	0.001	0.001	0.001	0.001
6	4.78±0.23[a]	5.25±0.38[a]	4.41±0.39[a]	40.16±2.03[b]	28.83±3.97[c]	20.08±1.18[c]	0.97	0.92	0.98	0.001	0.001	0.001	0.001	0.001	0.001
8	6.85±0.48[a]	6.36±0.60[a]	6.18±0.32[a]	25.66±3.12c	18.7±1.99[d]	11.61±0.85[d]	0.97	0.46	0.60	0.006	0.001	0.005	0.001	0.001	0.001
24	7.966±0.4[a]	5.26±0.66[a]	4.9±0.23[a]	10.06±0.62[de]	8.48±0.54[e]	6.08±0.55[de]	0.45	0.36	0.98	0.76	0.18	0.54	0.001	0.15	0.60
48	6.38±0.50[a]	6.1±0.38[a]	4.33±0.42[a]	6.2±0.71[e]	4.36±0.32[e]	4.01±0.29[e]	0.99	0.63	0.71	0.69	0.60	0.98	0.93	0.44	0.88

P values in the rows from ANOVA with double factorial, complete randomized design followed by Tukey's post hoc shows a pairwise comparison of water-born cortisol (ng L^{-1}) of *T. putitora* from three different rearing environments (barren, semi-natural and physically-enriched). Means with different superscript are significantly different ($P < 0.05$) in the columns compared water-born cortisol after stress with a basal level (pre-stress). Be-Pe= barren vs Physically-enriched, Se-Pe= semi-natural vs Physically-enriched, Se-Pe= semi-naturalvs Physically-enriched.

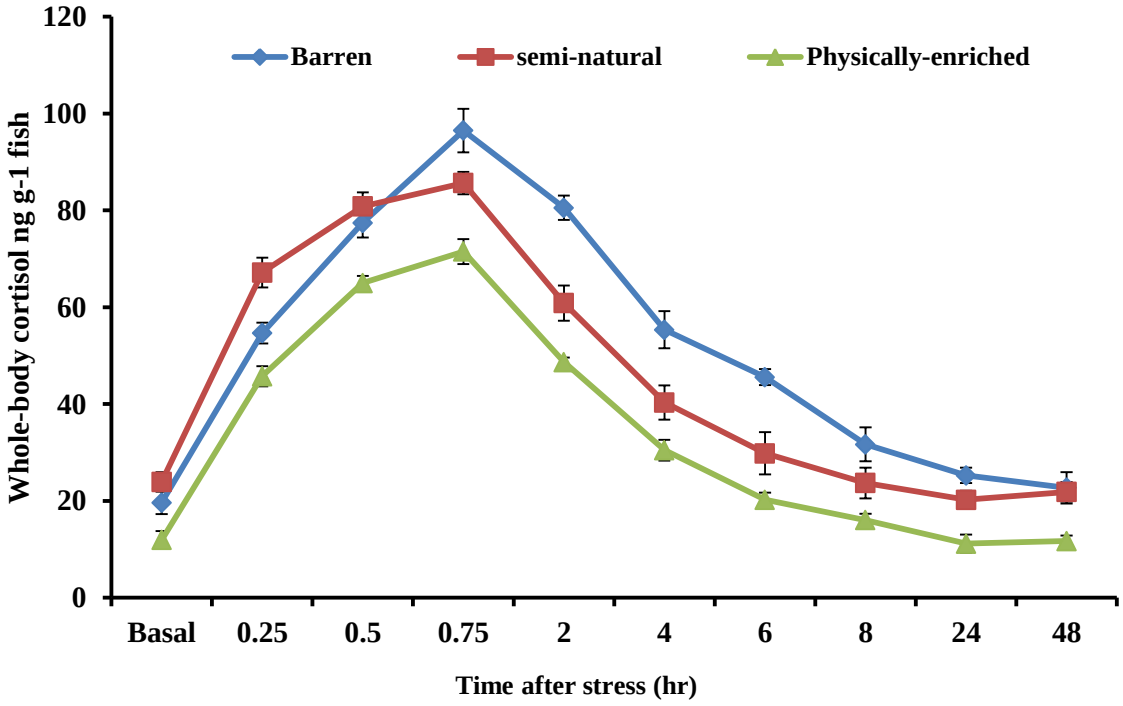


Fig. 2. Mean (± SEM) whole-body cortisol of barren, semi-natural and physically enriched environment reared *T. putitora* (n = 6) subjected to acute physical stress and sampled at various time intervals. Basal mean pre-stress level.

Table 3. Summary of the ANOVA comparing whole-body cortisol of barren, semi-natural and physically-enriched environment reared *T. putitora* during control and acute physical stress treatments.

Source of variation	df	SS	MS	F	p
Rearing groups	2	9660	4830	106.7082	< 0.001
Treatment	1	12353	12352.6	272.9058	< 0.001
Time	8	87150	10893.8	240.6765	< 0.001
Treatment: Rearing groups	2	397	198.6	4.3879	0.01406
Time: Rearing groups	16	3047	190.4	4.2074	0.001
Residuals	150	6789	45.3	-	-

Table 4. Mean (± SEM) whole-body cortisol (ngg^{-1} fish) of barren, semi-natural and physically-enriched environment reared *T. putitora* (n=6) subjected to acute physical stress and sampled at various time intervals. Basal mean pre-stress level.

Time (hr)	**Rearing groups**			**Statistics**		
	Barren	**Semi-natural**	**Physically-enriched**	**Be-Se**	**Be-Pe**	**Se-Pe**
				p	*p*	*p*
Basal	19.93 ± 2.37^{d}	23.93 ± 2.01^{d}	11.95 ± 1.85^{d}	0.52	0.05	0.004
0.25	54.66 ± 2.15^{c}	67.16 ± 3.06^{b}	45.75 ± 2.10^{b}	0.004	0.05	0.001
0.5	77.41 ± 2.99^{b}	80.83 ± 2.87^{a}	65.01 ± 1.45^{a}	0.60	0.007	0.003
0.75	96.5 ± 4.50^{a}	85.65 ± 2.31^{a}	71.5 ± 2.59^{a}	0.03	0.001	0.001
2	80.55 ± 2.5^{b}	60.85 ± 3.64^{b}	48.66 ± 0.92^{b}	0.001	0.001	0.006
4	55.33 ± 3.83^{c}	40.35 ± 3.53^{c}	30.48 ± 2.16^{c}	0.001	0.001	0.03
6	45.54 ± 1.64^{c}	29.83 ± 4.37^{cd}	20.25 ± 1.47^{cd}	0.001	0.001	0.05
8	31.66 ± 3.49^{ce}	23.7 ± 3.16^{d}	16.03 ± 1.28^{d}	0.09	0.001	0.17
24	25.25 ± 1.59^{d}	20.25 ± 1.87^{d}	11.03 ± 1.88^{d}	0.39	0.001	0.03
48	22.7 ± 3.24^{d}	21.86 ± 2.03^{d}	11.69 ± 1.18^{d}	0.97	0.01	0.02

P values in the rows from ANOVA with double factorial, complete randomized design followed by Tukey's post hoc shows apairwise comparison of cortisol level (ng/g fish) of *T. putitora* from three different rearing environments (barren, semi-natural and physically-enriched). Means with different superscript are significantly different ($P < 0.05$) in the columns compared cortisol level after stress with basal level (pre-stress). Be-Pe = barren vs Physically-enriched, Se-Pe = semi-natural vs Physically-enriched, Se-Pe= semi-natural vs Physically-enriched.

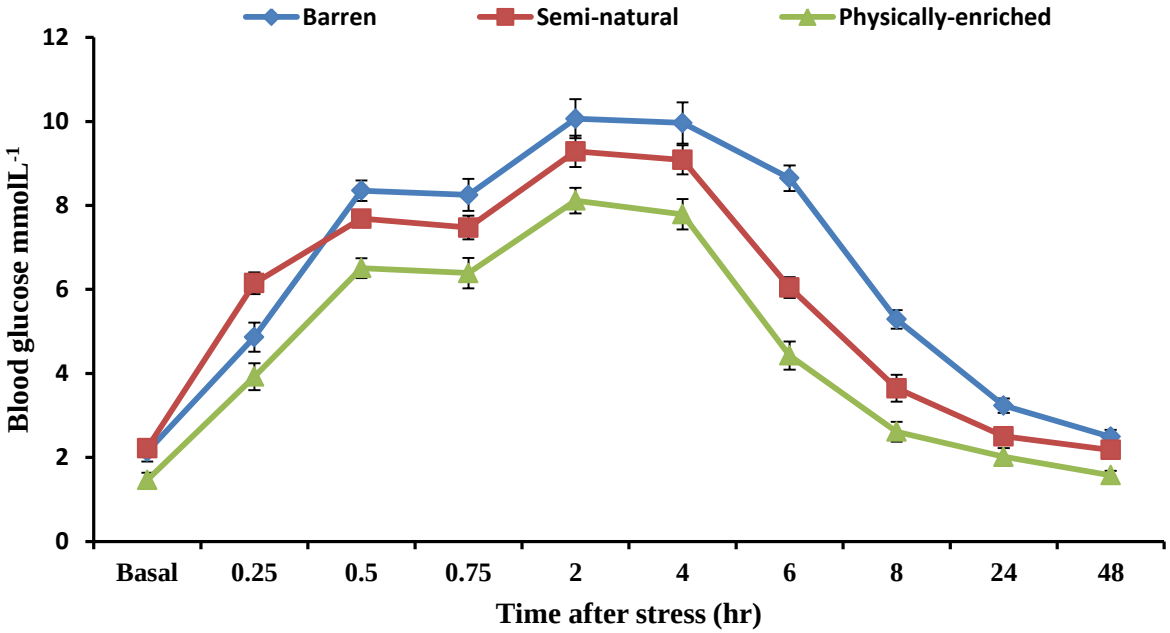


Fig. 3. Mean (± SEM) blood glucose (mmol L^{-1}) of barren, semi-natural and physically enriched environment reared *T. putitora* (n = 6) subjected to acute physical stress and sampled at various time intervals. Basal mean pre-stress level.

Table 5. Summary of the ANOVA examining the blood glucose level in barren, semi-natural and physically-enriched environment reared *T. putitora* during control and acute physical stress treatments.

Source of variation	df	SS	MS	F	p
Rearing groups	2	107.46	53.731	358.105	< 0.001
Treatment	1	251.89	251.888	1678.765	< 0.001
Time	8	987	123.375	822.259	< 0.001
Treatment: Rearing groups	2	5.04	2.521	16.799	0.001
Time: Rearing groups	16	37.48	2.342	15.612	< 0.001
Residuals	150	22.51	0.15		

Table 6. Mean (±SEM) blood glucose ($mmolL^{-1}$) in barren, semi-natural and physically-enriched environment reared *T. putitora* (n=6) subjected to acute physical stress and sampled at various time intervals. Basal mean pre-stress level.

Time (hr)	Rearing groups			Statistics		
	Barren	**Semi-natural**	**Physically-enriched**	**Be-Se**	**Be-Pe**	**Se-Pe**
				p	*p*	*p*
Basal	2.13 ± 0.23^{e}	2.222 ± 0.14^{e}	1.46 ± 0.17^{e}	0.92	0.008	0.002
0.25	4.86 ± 0.34^{c}	6.14 ± 0.25^{c}	3.92 ± 0.31^{c}	0.001	0.001	0.001
0.5	8.35 ± 0.24^{b}	7.68 ± 0.20^{b}	6.50 ± 0.23^{b}	0.009	0.001	0.001
0.75	8.25 ± 0.37^{b}	7.47 ± 0.28^{b}	6.38 ± 0.36^{b}	0.001	0.001	0.001
2	10.5 ± 0.46^{a}	9.28 ± 0.37^{a}	8.111 ± 0.30^{a}	0.001	0.001	0.001
4	9.96 ± 0.49^{a}	9.08 ± 0.34^{a}	7.78 ± 0.36^{a}	0.001	0.001	0.001
6	8.6 ± 0.30^{b}	6.04 ± 0.24^{c}	4.42 ± 0.33^{c}	0.001	0.001	0.001
8	5.28 ± 0.22^{c}	3.64 ± 0.31^{d}	2.61 ± 0.23^{d}	0.001	0.001	0.001
24	3.2 ± 0.17^{d}	2.50 ± 0.12^{e}	2.01 ± 0.20^{de}	0.001	0.001	0.07
48	2.49 ± 0.16^{e}	2.17 ± 0.12^{e}	1.57 ± 0.10^{e}	0.342	0.001	0.02

P values in the rows from ANOVA with double factorial, complete randomized design followed by Tukey's post hoc shows a pairwise comparison of blood glucose (mmol L^{-1}) of *T. putitora* from three different rearing environments (barren, semi-natural and physically-enriched). Means with different superscript are significantly different ($P < 0.05$) in the columns compared glucose after stress with a basal level (pre-stress). Bf-Sf= barren tank reared fish vs semi-natural reared fish, Be-Pe= barren vs Physically-enriched, Se-Pe= semi-naturalvs Physically-enriched, Se-Pe= semi-naturalvs Physically-enriched.

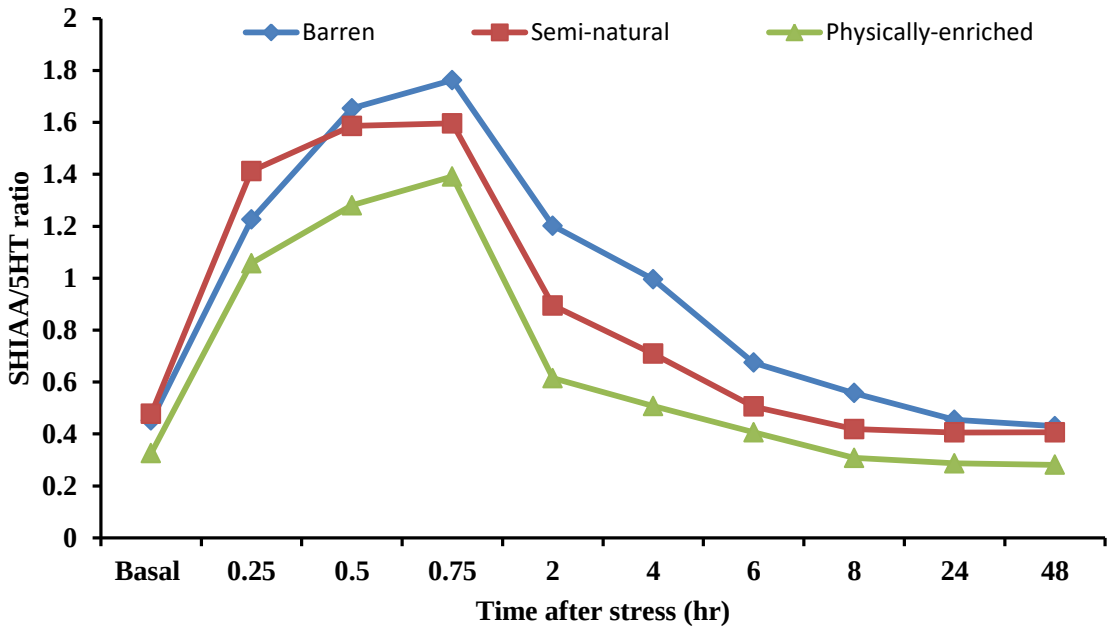


Fig. 4. Mean (± SEM) brain serotonergic activity (5HIAA/5-HT ratio) of barren, semi-natural and physically enriched environment reared *T. putitora* (n = 6) subjected to acute physical stress and sampled at various time intervals.. Basal mean pre-stress level.

Table 7. Summary of the ANOVA examining serotonergic activity (5HIAA/5-HT ratio) in the brain in barren, semi-natural and physically-enriched environment reared *T. putitora* during control and acute physical stress treatments.

Source of variation	df	SS	MS	F	p
Rearing groups	2	2.828	1.4139	48.8568	< 0.001
Treatment	1	3.108	3.1076	107.3827	< 0.001
Time	8	33.516	4.1895	144.7712	< 0.001
Treatment: Rearing groups	2	0.119	0.0598	2.0724	0.042713
Time: Rearing groups	16	1.041	0.065	2.2472	0.005973
Residuals	150	4.341	0.0289		

Table 8. Mean (± SEM) 5HIAA/5-HT ratio in barren, semi-natural and physically-enriched environment reared *T. putitora* (n=6) subjected to acute physical stress and sampled at various time intervals. Basal mean pre-stress level.

Time (hr)	Rearing groups			Statistics		
	Barren	**Semi-natural**	**Physically-enriched**	**Be-Se**	**Be-Pe**	**Se-Pe**
				p	*p*	*p*
Basal	0.45 ± 0.05^{d}	0.47 ± 0.03^{c}	0.32 ± 0.05^{d}	0.96	0.41	0.27
0.25	1.22 ± 0.04^{b}	1.47 ± 0.07^{a}	1.05 ± 0.08^{b}	0.05	0.23	0.001
0.5	1.65 ± 0.11^{a}	1.58 ± 0.05^{a}	1.28 ± 0.04^{ab}	0.77	0.001	0.01
0.75	1.76 ± 0.06^{a}	1.59 ± 0.03^{a}	1.39 ± 0.04^{a}	0.05	0.001	0.09
2	1.20 ± 0.13^{b}	0.89 ± 0.06^{b}	0.61 ± 0.08^{c}	0.001	0.001	0.01
4	0.99 ± 0.04^{b}	0.70 ± 0.05^{bc}	0.50 ± 0.06^{cd}	0.01	0.001	0.10
6	0.67 ± 0.07^{c}	0.50 ± 0.06^{c}	0.40 ± 0.05^{cd}	0.19	0.01	0.57
8	0.55 ± 0.08^{cd}	0.41 ± 0.07^{c}	0.30 ± 0.05^{cd}	0.33	0.03	0.52
24	0.40 ± 0.06^{d}	0.40 ± 0.05^{c}	0.28 ± 0.04^{d}	0.87	0.19	0.43
48	0.38 ± 0.10^{d}	0.40 ± 0.04^{c}	0.28 ± 0.05^{d}	0.99	0.60	0.68

P values in the rows from ANOVA with double factorial, the complete randomized design followed by Tukey's post hoc shows a pairwise comparison of the brain 5HIAA/5-HT ratio of *T. putitora* from three different rearing environments (barren, semi-natural pond and physically-enriched). Means with different superscript are significantly different ($P < 0.05$) in the columns compared the 5HIAA/5-HT ratio after stress with a basal level (pre-stress). Be-Se= barren vs semi-natural, Be-Pe= barren vs Physically-enriched, Se-Pe= semi-natural vs Physically-enriched.

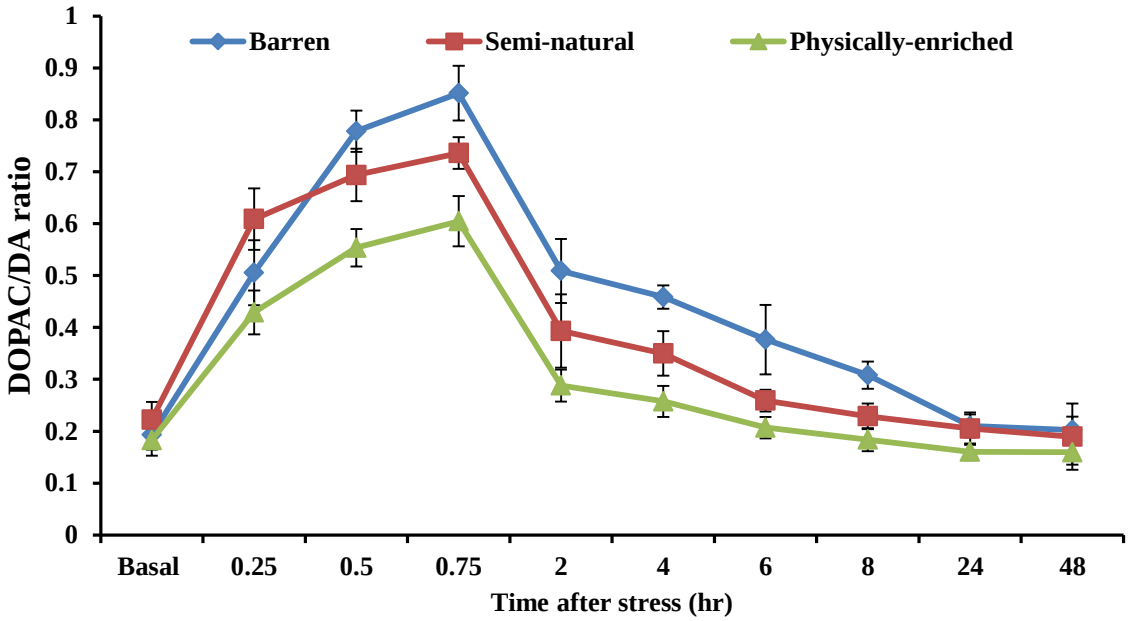


Fig. 5. Mean (± SEM) brain dopaminergic activity (DOPAC/DA ratio) of barren, semi-natural and physically enriched environment reared *T. putitora* subjected to acute physical stress (n = 6) and sampled at various time intervals. Basal mean pre-stress level.

Table 9. Summary of the ANOVA examining dopaminergic activity (DOPAC/DA ratio) in barren, semi-natural and physically-enriched environment reared *T. putitora* during control and acute physical stress treatments.

Source of variation	df	SS	MS	F	p
Rearing groups	2	0.5591	0.27957	30.7385	0.001
Treatment	1	0.6163	0.61633	67.7654	0.001
Time	8	5.7284	0.71605	78.7292	< 0.001
Treatment: Rearing groups	2	0.0533	0.02667	2.9318	0.05636
Time: Rearing groups	16	0.2256	0.0141	1.5504	0.0894
Residuals	150	1.3643	0.0091		

Table 10. Mean (± SEM) brain dopaminergic activity (DOPAC/DA ratio) in barren, semi-natural and physically-enriched environment reared *T. putitora* (n=6) subjected to acute physical stress and sampled at various time intervals. Basal mean pre-stress level.

Time (hr)	**Rearing groups**			**Statistics**		
	Barren tank	**Semi-natural**	**Physically-enriched**	**Be-Se** *p*	**Be-Pe** *p*	**Se-Pe** *p*
Basal	0.19 ± 0.02^{e}	0.22 ± 0.03^{bc}	0.18 ± 0.03^{c}	0.85	0.98	0.75
0.25	0.50 ± 0.06^{b}	0.60 ± 0.05^{a}	0.42 ± 0.04^{ab}	0.14	0.34	0.003
0.5	0.77 ± 0.04^{a}	0.69 ± 0.06^{a}	0.55 ± 0.03^{a}	0.27	0.001	0.03
0.75	0.85 ± 0.05^{a}	0.73 ± 0.03^{a}	0.60 ± 0.05^{a}	0.11	0.001	0.07
2	0.50 ± 0.06^{b}	0.39 ± 0.07^{b}	0.28 ± 0.03^{b}	0.09	0.001	0.13
4	0.45 ± 0.02^{bc}	0.34 ± 0.04^{bc}	0.25 ± 0.03^{bc}	0.12	0.001	0.21
6	0.37 ± 0.06^{bd}	0.25 ± 0.02^{bc}	0.20 ± 0.02^{c}	0.08	0.006	0.61
8	0.30 ± 0.03^{cde}	0.22 ± 0.03^{bc}	0.18 ± 0.02^{c}	0.32	0.06	0.69
24	0.21 ± 0.02^{de}	0.20 ± 0.02^{c}	0.16 ± 0.01^{c}	0.93	0.64	0.41
48	0.20 ± 0.03^{de}	0.18 ± 0.06^{c}	0.15 ± 0.02^{c}	0.96	0.71	0.84

P values in the rows from ANOVA with double factorial, the complete randomized design followed by Tukey's post hoc shows a pairwise comparison of brain DOPAC/DA ratio of *T. putitora* from three different rearing environments (barren, semi-natural and physically-enriched). Means with different superscript are significantly different ($P < 0.05$) in the columns compared the DOPAC/DA ratio of mahseer brain with a basal (pre-stress). Be-Se= barren vs semi-natural, Be-Pe= barren vs Physically-enriched, Se-Pe= semi-natural vs Physically-enriched.

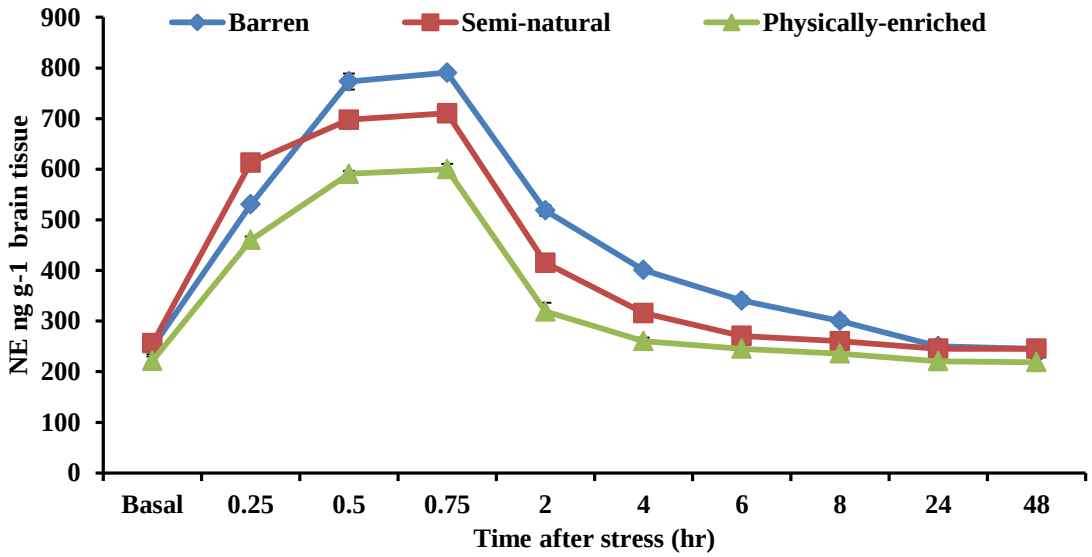


Fig. 6. Mean (± SEM) brain NE (ngg^{-1}) from barren, semi-natural and physically enriched environment reared *T. putitora* (n = 6) subjected to acute physical stress and sampled at various time intervals.. Basal mean pre-stress level.

Table 11. Summary of the ANOVA examining brain NE in barren, semi-natural and physically-enriched environment reared *T. putitora* during control and acute physical stress treatments.

Source of variation	df	SS	MS	F	p
Rearing groups	2	320306	160153	302.271	< 0.001
Treatment	1	469418	469418	885.972	< 0.001
Time	8	5038141	629768	1188.614	< 0.001
Treatment: Rearing groups	2	21867	10933	20.636	0.001
Time: Rearing groups	16	173501	10844	20.466	< 0.001
Residuals	150	79475	530		

Table 12. Mean (± SEM) NE (ngg^{-1} brain tissues) in barren, semi-natural and physically-enriched environment reared *T. putitora* (n=6) subjected to acute physical stress and sampled at various time intervals. Basal mean pre-stress level.

Time (hr)	**Rearing groups**			**Statistics**		
	Barren	**Semi-natural**	**Physically-enriched**	**Be-Se**	**Be-Pe**	**Se-Pe**
				p	*p*	*p*
Basal	249.094 ± 14.71^{e}	256.01 ± 9.28^{e}	221.65 ± 8.34^{d}	0.611	0.05	0.02
0.25	530.62 ± 7.28^{b}	612.949 ± 14.11^{b}	460.37 ± 7.01^{b}	0.001	0.001	0.001
0.5	773.41 ± 15.7^{a}	697.70 ± 13.6^{a}	590.75 ± 6.21^{a}	0.001	0.001	0.001
0.75	790.76 ± 15.78^{a}	710.37 ± 8.81^{a}	600.02 ± 10.59^{a}	0.001	0.001	0.001
2	518.56 ± 10.20^{b}	415.04 ± 9.66^{c}	319.12 ± 17.08^{c}	0.001	0.001	0.001
4	400.81 ± 6.57^{c}	315.80 ± 9.11^{d}	260.56 ± 6.85^{d}	0.001	0.001	0.001
6	340.60 ± 8.12^{d}	270.76 ± 8.61^{e}	245.51 ± 6.38^{d}	0.001	0.001	0.15
8	300.43 ± 9.02^{d}	260.09 ± 9.57^{e}	235.78 ± 4.59^{d}	0.009	0.001	0.18
24	250.49 ± 8.53^{e}	245.45 ± 13.11^{e}	220.6 ± 7.78^{d}	0.92	0.05	0.06
48	245.29 ± 7.39^{e}	245.36 ± 9.74^{e}	218.79 ± 6.76^{d}	0.92	0.05	0.05

P values in the rows from ANOVA with double factorial, complete randomized design followed by Tukey's post hoc shows a pairwise comparison of brain NE (ngg^{-1} brain tissue) of *T. putitora* of the three different rearing environments (barren, semi-natural and physically-enriched). Means with different superscript are significantly different ($P < 0.05$) in the columns compared NE after stress with basal level. Be-Se= barren vs semi-natural, Be-Pe= barren vs Physically-enriched, Se-Pe= semi-natural vs Physically-enriched.

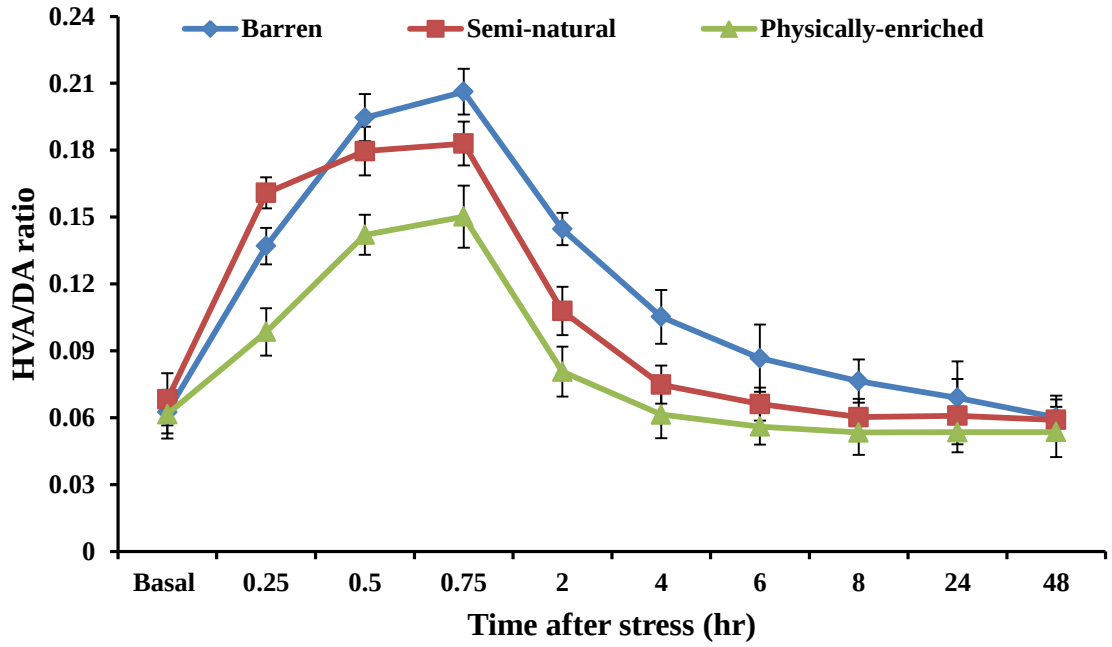


Fig. 7. Mean (± SEM) brain HVA/DA ratio of barren, semi-natural and physically enriched environment reared *T. putitora* (n = 6) subjected to acute physical stress and sampled at various time intervals. Basal mean pre-stress level.

Table 13. Summary of the ANOVA examining the HVA/DA ratio in barren, semi-natural and physically-enriched environment reared *T. putitora* during control and acute physical stress treatments.

Source of variation	df	SS	MS	F	p
Rearing groups	2	0.03603	0.018013	17.5027	0.001
Treatment	1	0.0247	0.024696	23.9963	0.001
Time	8	0.32214	0.040268	39.127	< 0.001
Treatment: Rearing groups	2	0.00399	0.001997	1.9406	0.1472
Time: Rearing groups	16	0.02242	0.001401	1.3616	0.1683
Residuals	150	0.15437	0.001029		

Table 14. Mean (± SEM) brain HVA/DA in ratio barren, semi-natural and physically-enriched environment reared *T. putitora* (n=6) subjected to acute physical stress and sampled at various time intervals. Basal mean pre-stress level.

Time (hr)	Rearing groups			Statistics		
	Barren	**Semi-natural**	**Physically-enriched**	**Be-Se**	**Be-Pe**	**Se-Pe**
				p	*p*	*p*
Basal	0.06 ± 0.009^{e}	0.06 ± 0.01^{c}	0.06 ± 0.01^{c}	0.99	0.92	0.96
0.25	0.13 ± 0.008^{bd}	0.16 ± 0.006^{ab}	0.09 ± 0.01^{bc}	0.47	0.02	0.001
0.5	0.19 ± 0.01^{ab}	0.17 ± 0.01^{a}	0.14 ± 0.009^{ab}	0.57	0.001	0.03
0.75	0.20 ± 0.01^{a}	0.18 ± 0.009^{a}	0.15 ± 0.01^{a}	0.31	0.01	0.15
2	0.14 ± 0.007^{bc}	0.10 ± 0.01^{b}	0.08 ± 0.01^{bc}	0.06	0.001	0.39
4	0.10 ± 0.01^{cd}	0.07 ± 0.008^{c}	0.06 ± 0.01^{c}	0.17	0.07	0.92
6	0.08 ± 0.01^{de}	0.06 ± 0.007^{c}	0.05 ± 0.008^{c}	0.66	0.19	0.65
8	0.07 ± 0.009^{e}	0.06 ± 0.008^{c}	0.05 ± 0.01^{c}	0.97	0.59	0.74
24	0.06 ± 0.01^{e}	0.06 ± 0.01^{c}	0.05 ± 0.005^{c}	0.99	0.85	0.83
48	0.06 ± 0.009^{e}	0.06 ± 0.009^{c}	0.05 ± 0.01^{c}	0.95	0.81	0.74

P values in the rows from ANOVA with double factorial, the complete randomized design followed by Tukey's post hoc shows a pairwise comparison of the HVA/DA ratio of *T. putitora* brain from three different rearing environments (barren, semi-natural pond and physically-enriched). Means with different superscript are significantly different ($P < 0.05$) in the columns compared brain DOPAC/DA ratio after stress with a basal level (pre-stress). Be-Se= barren vs semi-natural, Be-Pe= barren vs Physically-enriched, Se-Pe= semi-natural vs Physically-enriched.

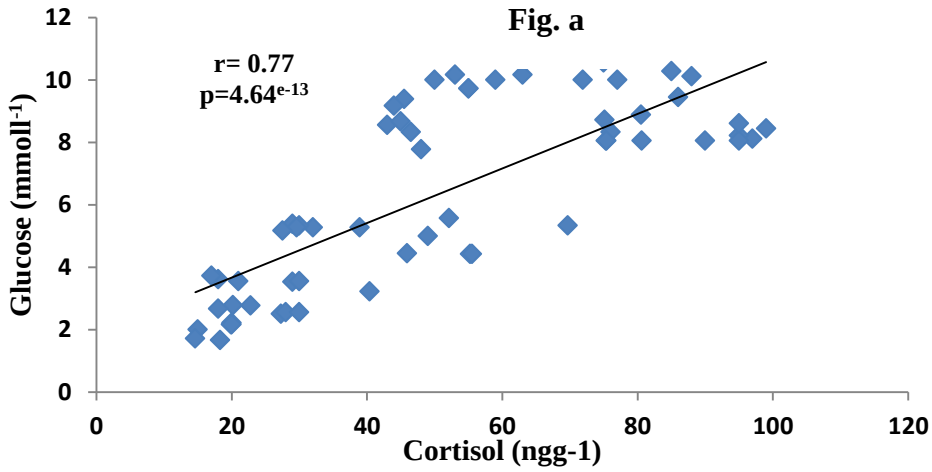


Fig. a
r= 0.77
p=4.64e-13
Glucose (mmoll-1)
Cortisol (ngg-1)
0
2
4
6
8
10
12
0
20
40
60
80
100
120

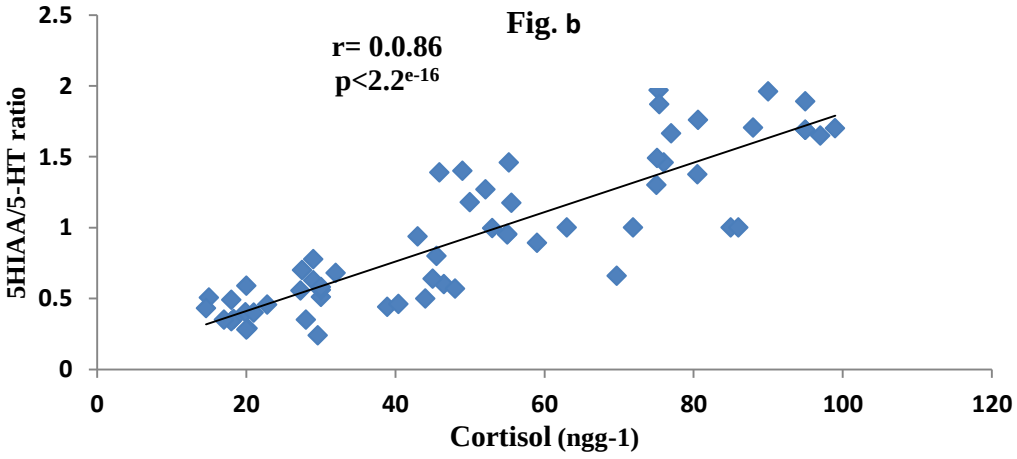


Fig. b
r= 0.0.86
p<2.2e-16
5HIAA/5-HT ratio
Cortisol (ngg-1)
0
0.5
1
1.5
2
2.5
0
20
40
60
80
100
120

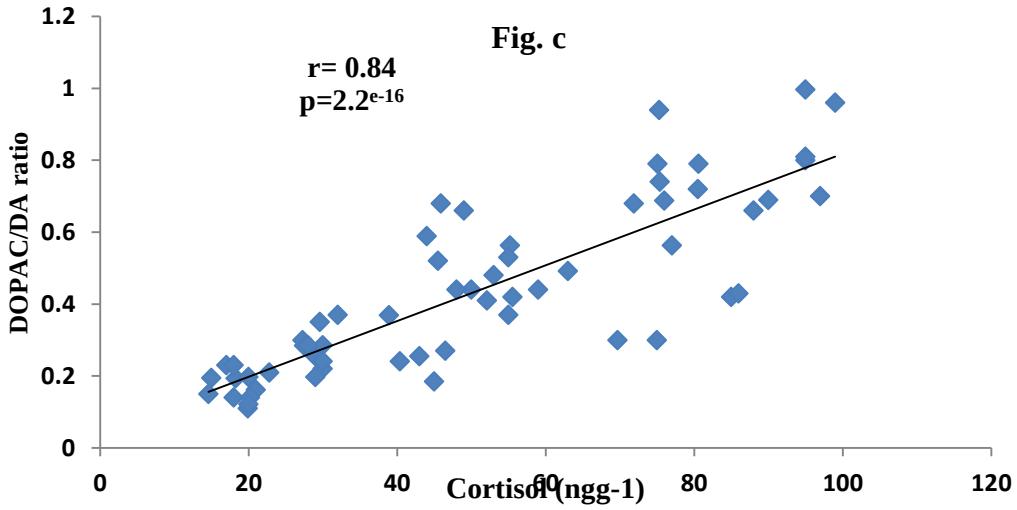


Fig. c
r= 0.84
p=2.2e-16
DOPAC/DA ratio
Cortisol (ngg-1)
0
0.2
0.4
0.6
0.8
1
1.2
0
20
40
60
80
100
120

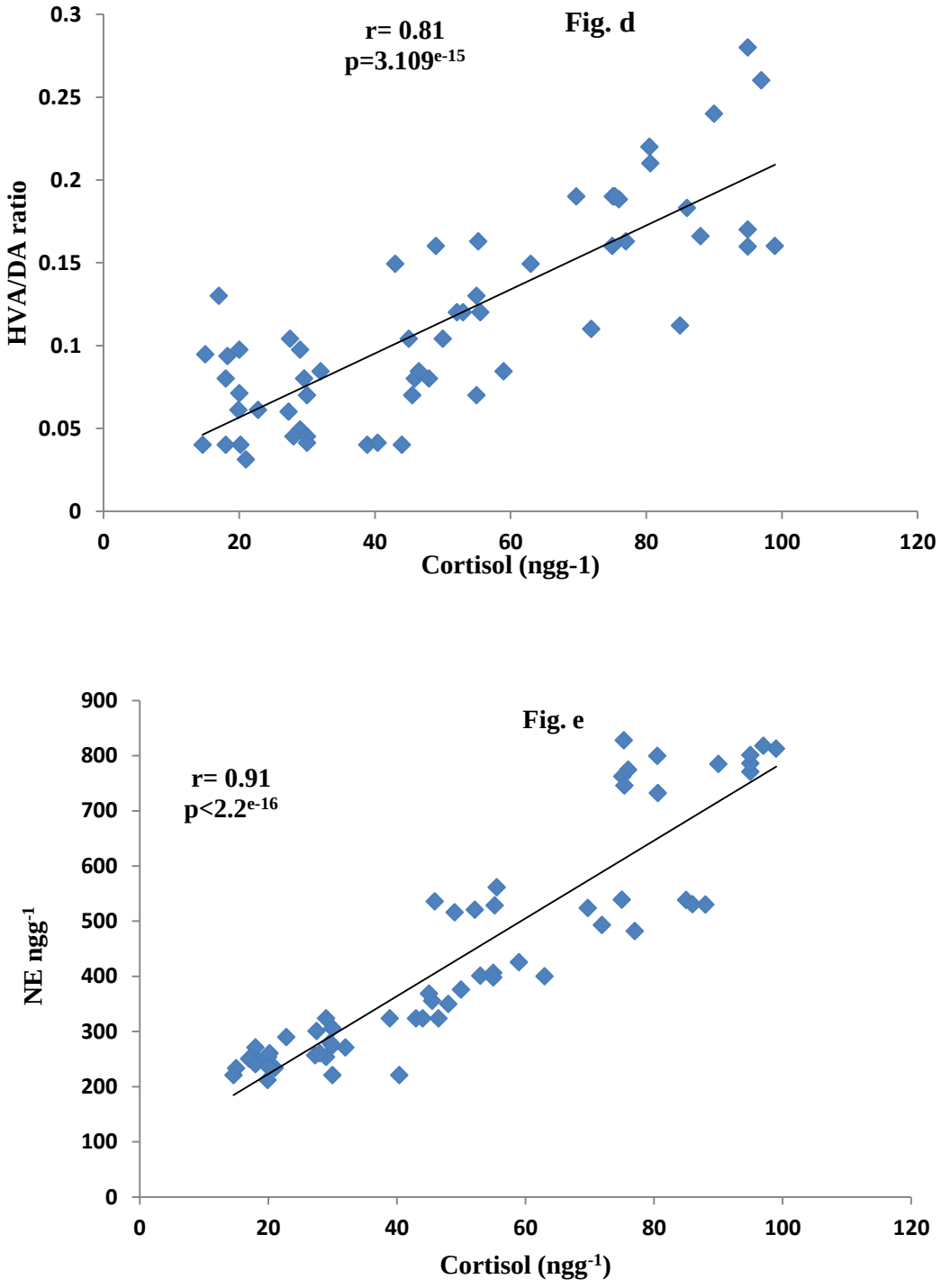


Fig. 8. The relationship of whole-body cortisol with glucose (a) 5HIAA/5-HT (b) DOPAC/DA (c), HVA/DA (d), NE (e) in barren reared *T. putitora* subjected to acute stress. Pearson's correlation r and p values are given.

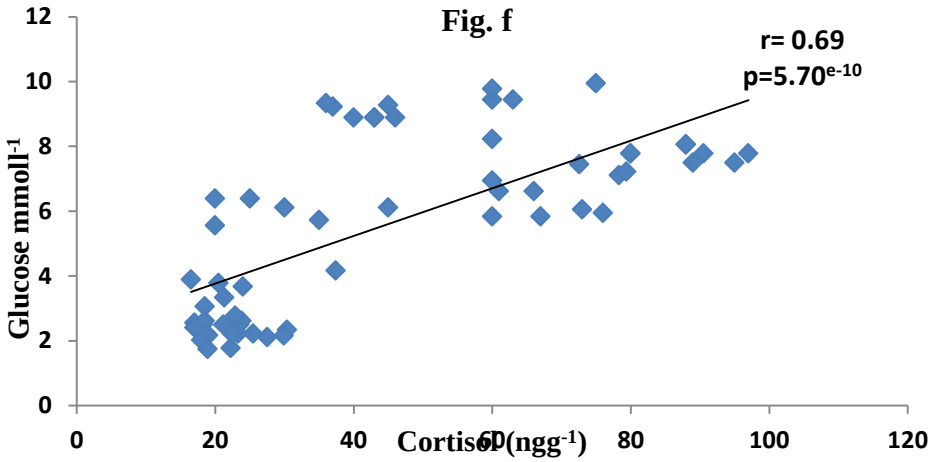


Fig. f
r= 0.69
p=5.70e-10
Glucose mmoll-1
Cortisol (nggˉ1)
0
2
4
6
8
10
12
0
20
40
60
80
100
120

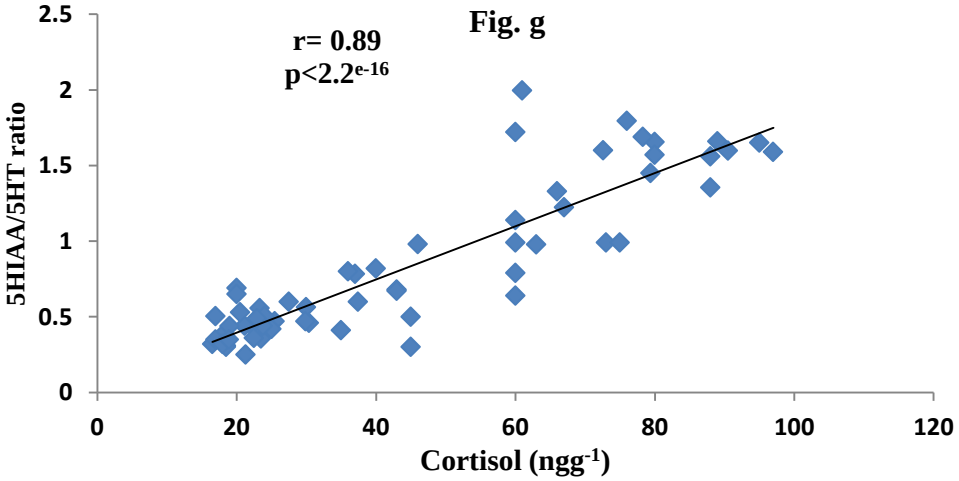


Fig. g
r= 0.89
p<2.2e-16
5HIAA/5HT ratio
Cortisol (nggˉ1)
0
0.5
1
1.5
2
2.5
0
20
40
60
80
100
120

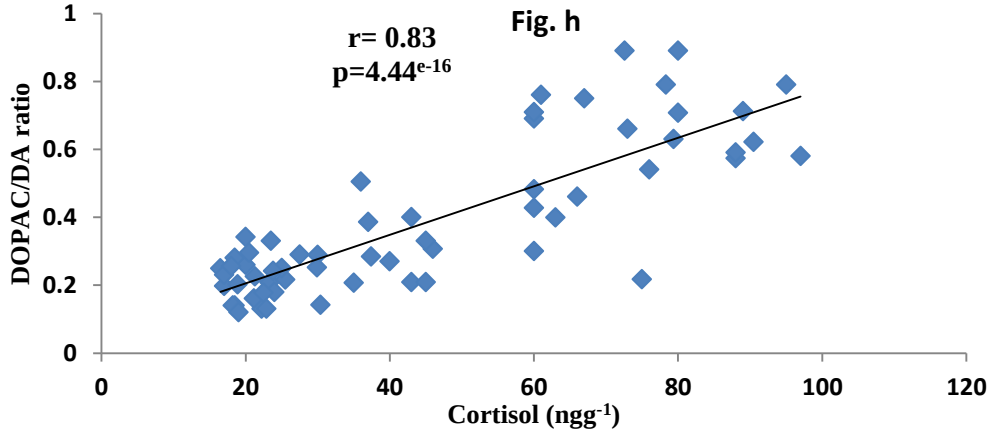


Fig. h
r= 0.83
p=4.44e-16
DOPAC/DA ratio
Cortisol (nggˉ1)
0
0.2
0.4
0.6
0.8
1
0
20
40
60
80
100
120

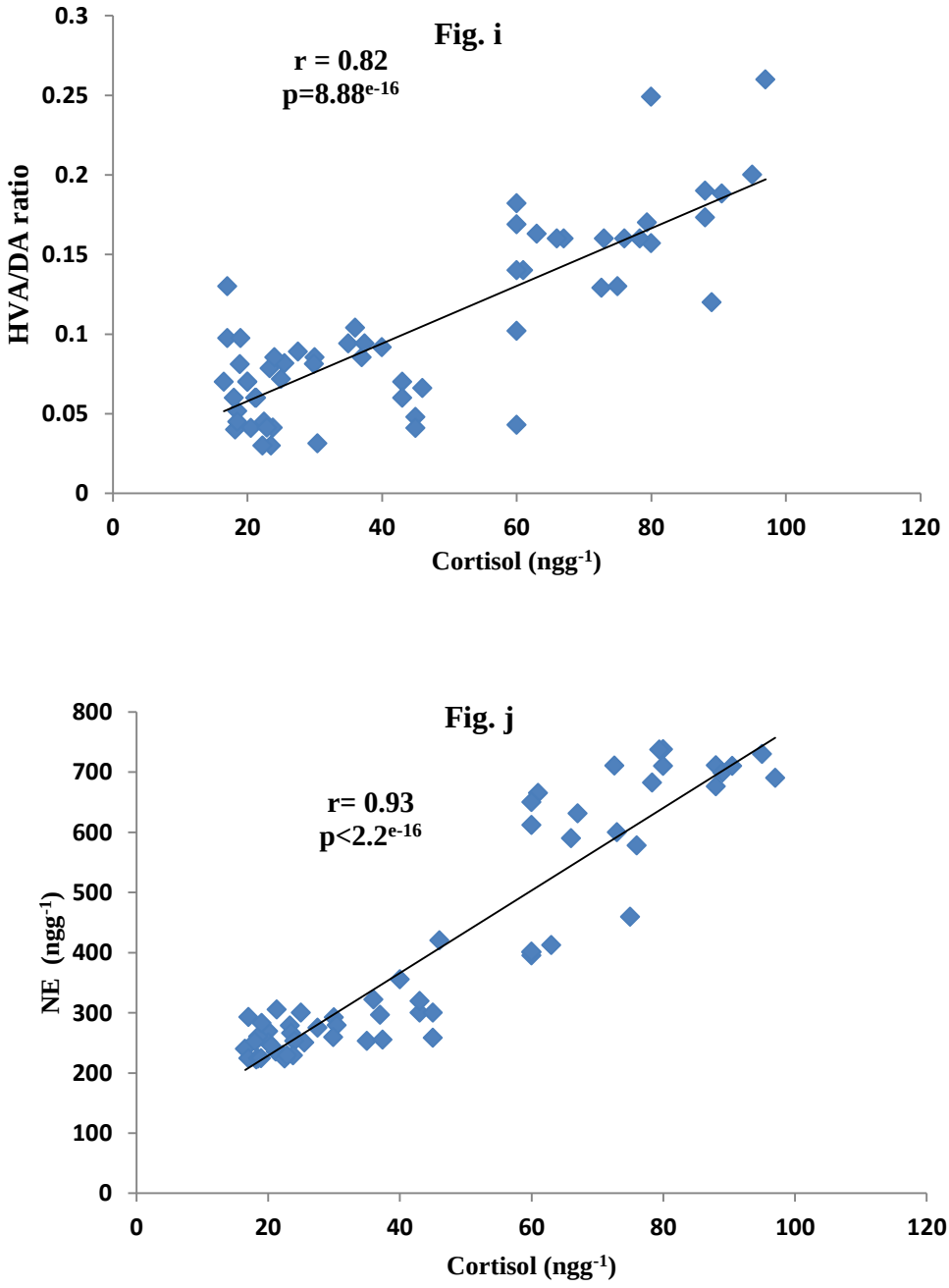


Fig. 9. The relationship of whole-body cortisol with glucose (f) 5HIAA/5-HT (g) DOPAC/DA (h), HVA/DA (i), NE (j) in semi-natural environment reared *T. putitora* subjected to acute stress. Pearson's correlation r and p values are given.

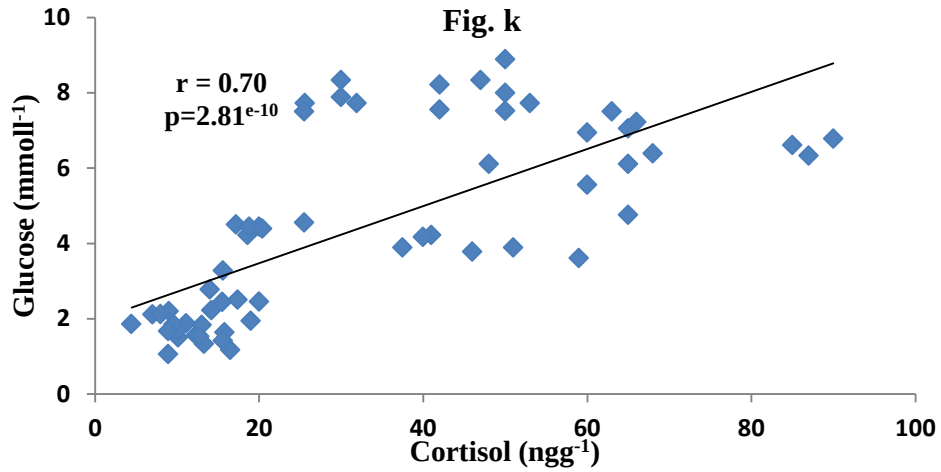


Fig. k
r = 0.70
p=2.81e-10
Glucose (mmoll-1)
Cortisol (ngg-1)
0
2
4
6
8
10
0
20
40
60
80
100

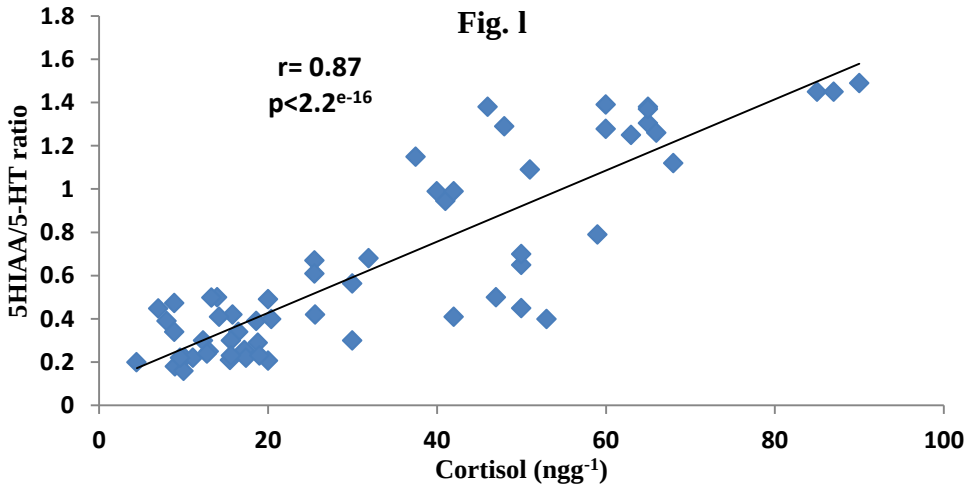


Fig. l
r= 0.87
p<2.2e-16
5HIAA/5-HT ratio
Cortisol (ngg-1)
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
0
20
40
60
80
100

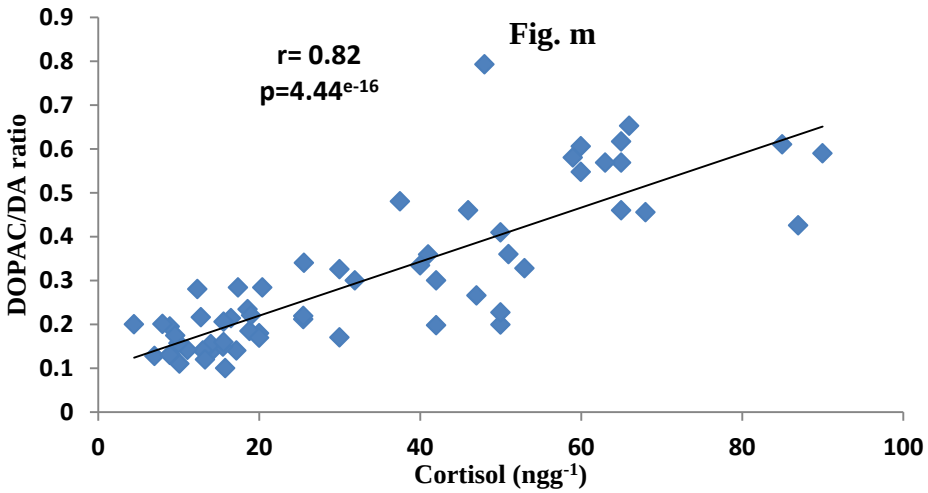
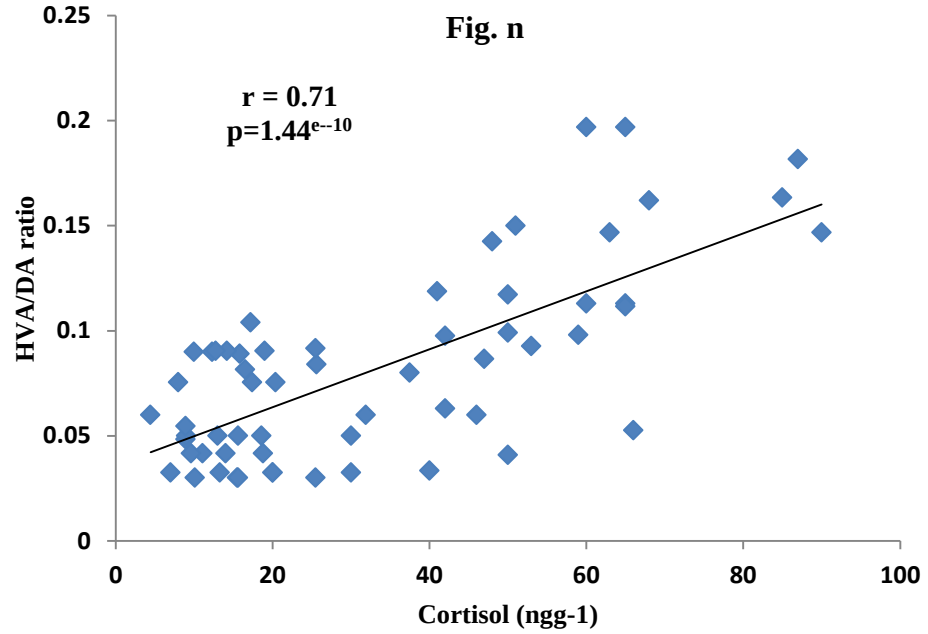


Fig. m
r= 0.82
p=4.44e-16
DOPAC/DA ratio
Cortisol (ngg-1)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
0
20
40
60
80
100



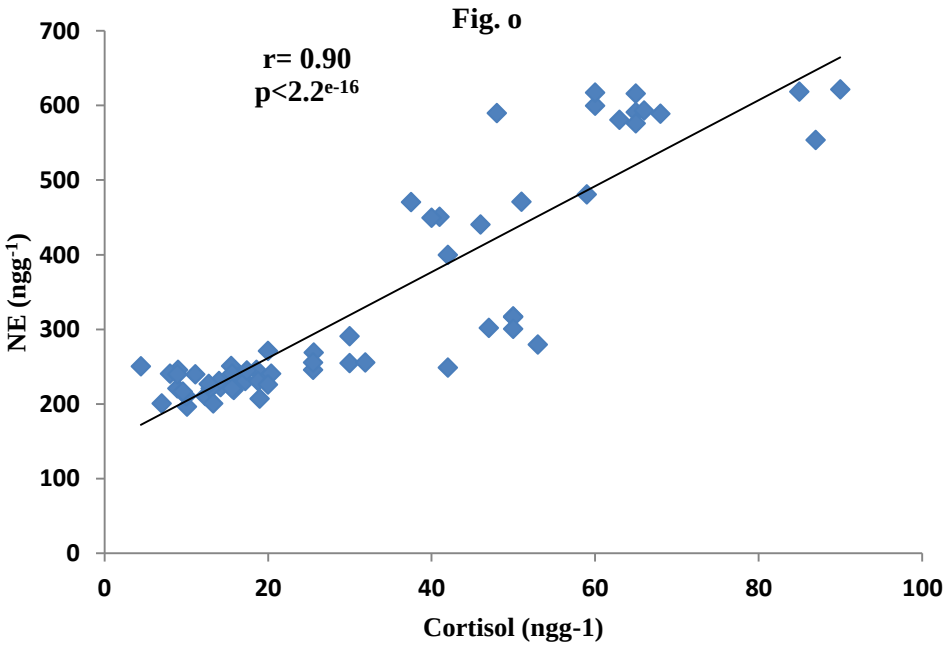


Fig. 10. The relationship of whole-body cortisol with glucose (k) 5HIAA/5-HT (l) DOPAC/DA (m), HVA/DA (n), NE (0) in a physically-enriched environment reared *T. putitora* subjected to acute stress. Pearson's correlation r and p values are given.

Table 15. Summary of relationship of whole-body cortisol with brain monoamenergic activity (monoamine/metabolite ratio) and blood glucose in acute stress treated *T. putitora* reared in barren, semi-natural and physically-enriched environment.

	Rearing Groups		
	Barren	**Semi-natural**	**Physically-enriched**
Glucose	r=0.77 p=4.645e-13	r=0.69 p=5.705e-10	r= 0.71 p=2.813e-10
Monoamine/metabolite ratio			
5HIAA/5HT	r= 0.86 p=<2.2e-16	r= 0.89 p=< 2.2e-16	r= 0.87 p=< 2.2e-16
DOPAC/DA	r= 0.84 p=< 2.2e-16	r= 0.83 p=4.441e-16	r=0.85 p=4.441e-16
HVA/DA	r=0.81 p=3.109e-15	r= 0.82 p= 8.882e-16	r= 0.71 p=1.441e-10
NE	r= 0.90 p=< 2.2e-16	r= 0.93 p=< 2.2e-16	r= 0.89 p=< 2.2e-16

Pearson's correlation r and p values are given, p value less than (0.05) show significant relationship. Relationship of whole-body cortisol with m onoamenergic activity and blood glucose are illustrated in figures 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22.

Table 16. Summary of relationship of whole-body cortisol with brain monoamenergic activity (monoamine/metabolite ratio**)** and blood blood glucose at different time intervals in acute stress treated *T. putitora* reared in barren, environment.

	Time (hr)									
	Control	0.25h	0.5h	0.75h	2h	4h	6h	8h	24h	48hr
Blood glucose	r= 0.77	r=0.35	r=0.22	r=0.42	r=-0.32	r=0.25	r=-0.59	r=0.06	**r=-0.91**	R=0.14
	p=0.66	p=0.48	p=0.22	p=0.39	p=0.52	p=0.62	p=0.21	p=0.89	**p=0.01**	P=0.78
Monoamine/metabolite ratio										
5HIAA/5HT	r= 0.44	**r= -0.89**	r= -0.29	r= -0.76	r= 0.11	r= -0.57	r= -0.49	r= -0.30	r= 0.60	r= 0.20
	p=0.56	**p=0.01**	p=-0.38	p=0.07	p=0.82	p=0.23	p=0.32	p=0.5	p=0.20	p=0.70
DOPAC/DA	r= 0.68	**r= -0.83**	r= -0.26	r= 0.50	r= -0.04	r= 0.19	r= 0.09	r= 0.58	r= 0.55	r= 0.62
	p=0.61	**p=0.03**	p=-0.26	p=0.31	p=0.93	p=0.71	p=0.86	p=0.22	p=0.25	p=0.18
HVA/DA	**r= -0.19**	r= 0.73	r= 0.95	r= -0.30	r= 0.36	r= 0.33	r= -0.34	r= -0.65	r= -0.30	r= -0.65
	p=0.002	p=0.09	p=0.95	p=0.55	p=0.47	p=0.52	p=0.50	p=0.15	p=0.56	p=0.15
NE	r= 0.57	r= -0.01	r= -0.18	r= 0.61	r= 0.57	r= 0.58	r= 0.34	r= 0.29	r= -0.42	r= -0.28
	p=0.73	p=0.97	p=0.56	p=0.19	p=0.23	p=0.22	p=0.50	p=0.57	p=0.39	p=0.57

Pearson's correlation r and p values are given, and significant relationships are indicated by bold font. p value less than (0.05) show significant relationship.

Table 17. Summary of relationship of whole-body cortisol with brain monoamenergic activity (monoamine/metabolite ratio**)** and blood glucose at different time intervals in acute stress treated *T. putitora* reared in a physically-enriched environment.

	Time (hr)									
	Control	**0.25h**	**0.5h**	**0.75h**	**2h**	**4h**	**6h**	**8h**	**24h**	**48hr**
Blood glucose	r=-0.17	r=-0.29	**r=0.82**	r=0.70	r=0.54	r=-0.41	r=-0.05	r=0.58	r=0.46	r=0.31
	p=0.74	p=0.57	**p=0.04**	p=0.11	p=0.26	p=0.41	p=0.92	p=0.21	p=0.35	p=0.54
Monoamine/metabolite ratio										
5HIAA/5HT	r=0.45	r=-0.38	r=-0.56	**r=0.91**	**r=-0.80**	r=-0.25	**r=0.90**	r=-0.75	r=-0.54	r=0.17
	p=0.36	p=0.45	p=0.24	**p=0.01**	**p=0.05**	p=0.62	**p=0.01**	p=0.08	p=0.26	p=0.74
DOPAC/DA	r=-0.11	r=0.54	r=-0.34	r=-0.63	r=0.062	r=-0.36	r=0.37	r=0.37	r=0.57	r=0.11
	p=0.83	p=0.25	p=0.50	p=0.17	p=0.90	p=0.48	p=0.46	p=0.46	p=0.23	p=0.83
HVA/DA	r=0.60	r=0.40	r=-0.03	r=0.25	r=0.35	r=0.20	r=-0.58	r=-0.29	r=0.64	r=-0.35
	p=0.20	p=0.42	p=0.94	p=0.63	p=0.49	p=0.70	p=0.22	p=0.56	p=0.16	p=0.49
NE	r= -0.30	r= 0.54	r= 0.14	r= -0.005	**r= -0.90**	r =-0.24	r= 0.50	r= -0.01	r= -0.34	r= -0.29
	p=0.55	p=0.26	p=0.78	p=0.99	**p=0.01**	p=0.64	p= 0.30	p=0.97	p=0.50	p=0.57

Pearson's correlation r and p values are given, and significant relationships are indicated by bold font. p value less than (0.05) show significant relationship.

Table 18. Summary of relationship of whole-body cortisol cortisol with brain monoamenergic activity (monoamine/metabolite ratio**)** and blood glucose at different time intervals in acute stress treated *T. putitora* reared in semi-natural environment.

	Time (hr)									
	Control	0.25h	0.5h	0.75h	2h	4h	6h	8h	24h	48hr
Blood glucose	r=-0.35	r=-0.73	r=-0.19	r=0.42	r=-0.29	r=-0.14	r=0.45	r=-0.14	r=-0.61	**r=-0.84**
	p=0.48	p=0.096	p=0.70	p=0.40	p=0.57	p=0.78	p=0.36	p=0.78	p=0.19	**p=0.03**
Monoamine/metabolite ratio										
5HIAA/5HT	r=0.58	r=-0.04	r=-0.57	r=0.15	r=0.071	**r=-0.84**	**r=-0.86**	r=0.71	r=0.37	r=0.62
	P=0.21	P=0.92	P=0.23	P=0.77	P=0.89	**P=0.03**	**3P=0.02**	P=0.10	P=0.46	P=0.18
DOPAC/DA	r=0.60	r=-0.04	r=-0.24	r=-0.57	r=-0.17	r=-0.58	r=-0.72	r=0.37	r=-0.38	r=0.25
	P=0.20	P=0.93	P=0.63	P=0.22	P=0.73	P=0.22	P=0.10	P=0.45	P=0.45	P=0.61
HVA/DA	r=0.63	r=-0.08	r=-0.11	**r=0.90**	r=0.51	**r=-0.91**	r=-0.35	r=0.70	r=-0.61	r=-0.05
	P=0.17	P=0.87	P=0.82	**P=0.01**	P=0.29	**P=0.00**	P=0.48	P=0.11	P=0.19	P=0.91
NE	r=0.60	r=-0.67	r=0.53	r=-0.01	r=0.53	r=-0.20	r=-0.19	r=0.01	r=0.33	r=0.01
	P=0.20	P=0.14	P=0.27	P=0.98	P=0.27	P=0.70	P=0.70	P=0.97	P=0.51	P=0.97

Pearson's correlation r and p values are given, and significant relationships are indicated by bold font. p value less than (0.05) shows significant relationship.

4. Discussion

In the current experiments, physical enrichment in the form of stones, PVC pipes and vegetation showed certain positive effect on HPI axis and brain dopaminergic and serotonergic systems and clearly differentiate the physically enriched group of fish from barren and semi-natural groups after exposure to acute stress. Fish from barren and the semi-natural environment reared groups had somewhat higher levels of all studied stress parameters (whole body and water borne cortisol, blood glucose and brain monoamines as compared to physically enriched reared group. These differences in basal levels may be due to the presence of unpredictable stressors in the rearing environments of barren and semi natural reared groups (Ladewig, 2000). However, overall slightly low basal levels of stress hormones in physically enriched groups could be due to the presence of shelter or hiding place t. It seems that structural enrichment regulates the external disturbance and lower conspecific aggression (Batzina et al., 2012, 2014).

Cortisol is extensively used as stress indicator. It shows an elevated level under acute or chronic stress (Barton and Iwama, 1991; Pickering, 1998; Sumpter, 1997; Wendelaar Bonga, 1997; Barton, 2002; Ramsay et al., 2006; Zuberi et al., 2011, 2014). Here we also used cortisol for the determination of enrichment on the physiological stress response of mahseer. Although, mostly cortisol is determined by circulating cortisol concentrations (Barton et al., 2005) but here the fish are small to collect blood for analysis. To overcome the limitation of insufficient blood, whole body and waterborne cortisol was determined. Many studies analysed whole body cortisol and suggested an appropriate indicator of stress (Pottinger et al.,2002; Ramsay et al., 2006; Zuberi et al., 2014). The significantly low basal whole body cortisol concentration in the physically enriched reared group of fish (Table 4) reflects the reduce stress and beneficial impact of rearing enrichment. It appears that rearing environment is appropriate for mahseer.

For endangered and small fish, measurement of cortisol that is released through gills in surrounding water is an alternative way of evaluating stress (Scott and Ellis, 2007; Scott et al., 2008; Zuberi et al., 20011). Although, in present study, pre-stress whole body cortisol concentration in a physically enriched group showed

significant difference with other rearing groups but water- borne cortisol did not clearly signify this difference. This may be due to sampling procedure or rearing environment exposure. It is suggested that type of enrichment, exposure duration and experimental procedure may influence the results (Näslund et al., 2013). Like our results , in zebrafish (*Danio rerio*) enrichment either slightly increased concentrations of cortisol (von Krogh et al., 2010) or did not show any significant effect (Wilkes et al., 2012).

Like whole body cortisol, the basal levels of blood glucose also clearly differentiate the physically enriched group from other two rearing groups. The significantly higher basal level of blood glucose in a barren reared group of fish reflects the presence of intermittent stressors in the rearing environment. It seems that in structure less/featureless environment, fish don't have any place to avoid external disturbance while low level in the physically enriched group might be related to reduce stress because of shelter (hiding place). Among monoamines, except NE basal all others (5HIAA/5HT, DOPAC/DA and HVA /DA ratios) did not show any significant difference among different rearing groups.

Scanty of literature is available on the impact of rearing environments on brain monamines in fish. To our knowledge only two studies reported the decrease in brain monoamines, when crucian carp *Carassius carassius* (Höglund et al., 2005) and gilthead seabream *Sparus aurata* (Batzina et al., 2014) reared in enriched environments. It is reported that presence of hiding material in the rearing environment, reduced the serotonergic activity in the brain stem and optic tectum of *C. carassius* (Höglund et al., 2005). Moreover, Blue or Red-Brown substrates in the rearing environment of gilt-head bream (*S. aurata*) reduced the 5-hydroxyindoleacetic acid (5-HIAA) and serotonin levels (Batzina et al. (2014). In contrast to our study, physical enrichment did not alter the NE level in *S. aurata* . The variable results might be related to substantial differences in species and population (Valdimarsson et al., 2000).

In contrast to basal levels, post stress response over time clearly differentiated between different rearing groups (Tables 2, 4, 6, 8, 10, 12, 14, Figurs. 1 to 7). The initial trend of stress response was more rapid in semi-natural pond reared fish compared with barren and physically-enriched reared advance fry of mahseer.

However the recovery rate from acute handling stress was almost similar in semi-natural pond and physically-enriched tank reared mahseer. In all trials, barren reared tank mahseer shows more prolong stress response and recovery rate from acute stress than semi-natural pond and physically-enriched tank reared fish. On contrary to our results, after acute stress cortisol levels were similar in barren and physically reared groups of Atlantic salmon, *Salmo salar* (Näslund et al., 2013), suggesting that barren environment not blunt the stress response. Our clear difference in stress response may be due to the protocol used for studying stress response. Here we study the stress response over time after exposing 5 min chasing and 2 min confinement as compared to Näslund et al. (2013) where cortisol was measured at specific time, i.e. after 30 min confinement. Both whole body and water borne cortisol showed significant difference in magnitude at peak levels among different rearing groups, low in physically enriched reared group in comparison to other groups. Moreover, the same group showed rapid recovery from stress. The stress response latencies are related to lifestyle differences (Vijayan and Moon, 1994; Wright et al., 2007). Here it appears that physically enrichment i.e shelters reduced the metabolic demand in mahseer which may be due to reduce stress.

In addition to cortisol, blood glucose also increased after acute stress in all rearing groups and indicate the activation of HPI axis. Blood glucose also showed significant differences in magnitude in all rearing groups over time. Barren rearing showed an increase blood glucose level compare to other groups indicated the increase demand of energy (Begg and Pankhurst, 2004). Although, in the present study, post stress elevation of brain serotonergic activity (5HIAA/5HT ratio), dopaminergic activity (DOPAC/DA and HVA /DA ratios) and Norepinephrine (NE) levels and significant difference in the magnitude of these parameters were observed among rearing groups but no study is available to compare our results. The available study only reported the effect of enrichment on the basal level of brain monoamines *S. auratus*, (Batzina et al., 2014) *C. carassius* (Höglund et al., 2005).

The role 5-HT in the regulation of HPI axis has been suggested previously (Winberg et al., 1997) in rainbow trout (*Oncorhynchus mykiss*). The positive correlation between whole body cortisol and brain monamines in the present study suggest the role of monoamines in regulation of stress responses. It seems that the

5HT integrated stress response in response to a stressor (Øverli et al., 2005; Winberg et al., 1992). In the present study, increase levels of monoamines (DOPAC/DA and HVA /DA ratios and NE levels in response to acute stress and their positive relation with cortisol (Fig 4 to 7) indicated the influence of monoamines in the regulation of HPI axis and suggested that these parameters could be used while considering the welfare of fish. These parameters are very well studied in mammals (Herman and Cullinan, 1997; Torres et al., 2002) and need to be studied in depth in fish for understanding the lying mechanism of physiological stress response and behaviour of fish.

It has been reported that rearing of crucian carp *C. carassius* in aquaria with substrate as a hiding material, led to reduced serotonergic activity in the brain stem and optic tectum (Höglund et al., 2005). It has been also observed that mice and rats reared in an enriched environment, had reduced brain serotonin system activation (i.e. Reduced 5-HT, 5-HIAA and/or 5-HIAA/5-HT) as well as reduced DA levels in specific brain areas (Brenes et al., 2008; McQuaid et al., 2012). Batzina et al. (2014) reported that fish reared in blue or red-brown substrate has comparatively low serotonergic activity and DA levels compared to fish reared in green substrate and or reared without substrate. It has been previously reported that lack of hiding material led to an elevation of 5-HT activity in the brain stem and the optic tectum compared to fish with available hiding material when exposed to predator skin extract (Höglund et al., 2005). These data are in line with present brain neurotransmitters results for fish reared physically-enriched and semi-natural pond environment.

5. Conclusions

In conclusion, our main results suggest that manipulation of rearing environment (physical enrichment) in the form presented here can reduce basal stress levels as indicated by whole-body cortisol, water-born cortisol, blood glucose and provide hatchery-reared mahseer with a more advantageous stress coping strategies. Furthermore, fish reared in physically-enriched environment displayed lower post-stress brain 5-HIAA/5-HT and DOPAC/DA ratios and central NE than barren and semi-natural environment reared mahseer. These results have important implications for a possible way of improving the welfare of mahseer by improving rearing

environment, i.e. to provide substrate or hiding place in rearing tanks. It will help to fulfil the physiological demand of fish and reduce their stress level. Overall provide information, how mahseer stress sensitivity can be modified by environmental enrichment and prepare fish to cope stressful wild environment.

CHAPTER # 4

Effects of Enrichment on the Life Skills Development of Endangered Fish Mahseer (*Tor putitora*).

ABSTRACT

Structural enrichment in the rearing environment, not only promotes fish welfare, but also affect several aspects of behavioural biology of fish in aquaculture. Here an attempt was made to use physical-enrichment to improve the behaviour of hatchery-reared fish.In this study, three groups of 15 days old mahseer (*Tor putitora*) hatchlings were reared up to advanced fry stage in barren (without any substrate), physically-enriched (gravel bed, substrate and plants) and semi-natural environments (earthen pond having a natural feed) respectively and the behavioural profiles of fish from these environments were compared. We illustrate that increased structural complexity during early life significantly affect various behavioural characteristics of the fish. Exploratory behaviour, predation and anti-predatory response was significantly ($p < 0.05$) higher in fish reared in physical enrichment and semi-natural environment than in barren-reared fish. These results have important implications for a possible way of improving the outcomes of restocking program of endangered fish species by modifying conventional hatchery-rearing environment

1. Introduction

Conventional hatchery rearing environments are lacking natural key stimuli important for the development of natural behaviour. This can severely affect the natural foraging, predatory and anti-predatory behaviour of animals, and become detrimental in wild environment where the animal is highly motivated to carry out particular behaviours (Reinhardt, 2004; Dawkins, 2006). Generally, fish are reared and grow in earthen, semi earthen and concrete ponds in static, featureless, predator free environments at high densities on prepared feed, which prevents them from learning how to behave in their wild environment. The hatchery rearing environment provide no structure or shelter, which in the wild is important as defence against predators and it has been shown that adding shelter to the rearing environment can decrease metabolic demands and stress levels (Millidine et al., 2006; Näslund et al., 2013). Also, fish in a hatchery environment have high growth rate, which has recently been connected to shorter memory duration (Brown et al., 2011). On the other hand, wild fish live in more diverse environments and learn by experience how to capture and handle various live preys (Sundström and Johnsson, 2001).

Several actions have been proposed as solutions to ease the foraging, predator avoidance and reproductive behavioural deficiencies in hatchery reared fish, e.g., life-skills training, social learning protocols, and acclimatization before release, and environmental modification (Brown and Day, 2002). Generally, to improve the animal welfare, environmental enrichment is mainly used in the zoo and laboratory animals, as compare to domesticated animals (Young, 2003). However, recent investigations on many fish species suggest that enrichment of rearing environment can promote behavioural adaptability (Braithwaite and Salvanes, 2005) and foraging abilities (Brown et al., 2003; Strand et al., 2010; Rodewald et al., 2011) and more complex rearing environments promote the development of fish brains (Näslund et al., 2012), cognition (Brown et al., 2003; Strand et al., 2010), boldness (Roberts et al., 2011) and survival in the wild environment after restocking (Maynard et al., 1996). Additionally, enrichment also influence social interaction (Salvanes and Braithwaite, 2005) and reduce anxiety (Maximino et al., 2010). Studies on rodent have revealed that behavioural and neural plasticity and the development of cognitive abilities are

influenced positively by increasing the structural complexity of the rearing conditions (van Praag et al., 2000).

Worldwide, restocking programs has been commonly used in attempts to minimize the effects of over-fishing, environmental degradation and conservational failure (Salvanes, 2001; Myers et al., 2004). Survival rates in many restocking programs are low for newly released hatchery reared fish (Tsukamoto et al., 1997). For successful restocking program, fish rearing conditions should be modified to make fish as adaptive as possible to minimize mortality rates upon release into the wild environment (Brännäs and Johnsson, 2008). Physical and social enrichment in the rearing environment strongly enhances the ability of fish to adopt important life skills such as exploratory behaviour, ability to feed on natural prey, and the ability to avoid predators (Braithwaite and Salvanes, 2008). Increased environmental complexity has been proven to promote behavioural and neuronal plasticity and learning in fish. These adaptations will further increase the survival of fish after release into the wild for restocking purposes (Spence et al., 2011; D'Anna et al., 2012).

Mahseer is a world famous, high market valued game and food fish of South Asian countries, including Pakistan, distributed in most of the trans-Himalayan region, ranging from Afghanistan to Myanmar (Desai, 2003; Singh et al., 2009). It inhabits the river, lakes and reservoirs and prefers high-oxygenated, clear water with rocky bed habitat. It is sensitive to low temperature, thus congregates by hundred during winter (Desai, 2003). Mahseer is a local migrant, shows short distance migration for feeding and breeding purposes. Generally breed during floods and spawn over rocky, gravel substrates (Mohan, 2000; Nautiyal et al., 2007). It is a marginal bottom feeder and shows variation in dietary habit, as to increase in size. Fry are carni-omnivorous, feed on diatoms filamentous algae and insects, juvenile are insectivorous, while adult feed on filamentous algae, aquatic macrophyte and benthic animals (Dinesh et al., 2010).

More recently, there has been an increase in anthropogenic threats to the natural populations of mahseer, which can affect their survivability (Hussain and Hossain, 1999). Depletion of natural populations of mahseer has been reported from

various parts of the world, including, Pakistan (Mirza et al., 1994), India (Nath et al., 1994), Papua New Guinea (Coates, 1991), Nepal (Shrestha, 2002), Turkey (Balik, 1995) and Bangladesh (Hussain and Mazid, 2001). Mahseer is now identified as an endangered species (IUCN, 2016). Scientists have suggested that special consideration is required to protect this fish species from future elimination and extinction (Hussain and Mazid, 2001; Islam, 2002; Rahman et al., 2005).

The early rearing environment of fish can be constructed to provide living conditions that favour the development of more adaptive foraging, exploratory, anti-predatory etc. behaviours. We therefore hypothesized that providing hatchery reared mahseer with an enriched environment could promote specific behaviours (e.g. anti-predatory, exploratory, foraging behaviour etc.), essential to cope with life threatening challenges (searching and competition of food, predation risk etc.). To test this hypothesis, we devised three different rearing environments that contrasted in their levels of complexity and heterogeneity. Mahseer hatchlings were reared up to advanced fry stage in these three different rearing environments and the behavioural profiles of fish from these environments were compared.

2. Material and methods

2.1. Experimental Animals

Tor putitora 15 days, hatchlings (10-15mm; after semi-quiescent stage), about 25000 in number, were collected from a breeding tank (barren concrete circular tank) of Fish Hatchery Hattian Attock, Pakistan, and transported in oxygen filled plastic bags (36cm length × 24cm width; 10 L water) to the Fisheries and Aquaculture Research Station, Department of Animal Sciences, Faculty of Biological Sciences, Quaid-I-Azam University Islamabad, Pakistan.

2.3. Manipulation of rearing environment

To investigate the effect of early rearing environment on exploratory, predatory and anti-predatory behaviours, the hatchlings of (*Tor putitora*) were housed in three different rearing environments, 1) Barren: Rectangular fibreglass tanks (120cm length × 60cm width × 60cm height) without any substrate (Clear bed), while plain contained only an aerator. 2) Physically enriched: Similar size fibreglass rectangular tanks (120cm length × 60cm width × 60cm height) with 1cm thick 1.0 to 1.5 cm diameter gravel bed, 4 plastic plants having 12 cm height, 2PVC pipes (10 cm length; 4 cm diameter and 5cm length; 4 cm diameter and aerator = 3) Semi-natural :250 m^2 earthen pond (20m × 12.5m) with concrete walls, having natural food organisms (phytoplankton, zooplankton, diatoms, some protozoa etc.) but without common aquatic weeds or other substrate. Before stocking, the ponds were limed (calcite lime; $CaCO_3$; 49.42 kg/100 m^2, fertilized with organic (Animal manure; cow dung 8 kg/100 m^2/week) and inorganic (Nitrogen fertilizers; 0.5kg/100 m^2/week; phosphate fertilizers; 0.25 kg/100 m^2/week) fertilizer, thus to provide a sufficient amount of natural food organisms (A conventional technique in Pakistan for the rearing of mahseer). Replicate of three for barren and physically enriched and two for semi-natural environments were maintained and hatchlings were stocked at the rate of 200 hatchlings per tank and about 10,000 hatchlings per semi-natural pond. Initially, low water level (about 0.6 m in each earthen pond and 200L in fibreglass tanks maintained, but after two months raised to 1 m and 350 L respectively. All

experimental groups were supplied with water from a nearby freshwater stream (Rumli freshwater stream). Hatchlings in barren and physically enriched groups were initially fed commercially available prepared powder feed (Oryza organics fish feed, powder; 55% crude protein, 12% fats, crude fiber 2% and 10% moisture) after every second hour, then gradually changed from powder feed (first two months) to crumbled (after two months) and then sinking pellets (last one month) (Oryza organics fish feed, size 2mm; 45% crude protein, 14% fats, 2% crude fiber and 10% moisture), at the rate of 4% body weight twice per day. In the ponds sufficient natural food organisms were maintained with the aid of fertilizer. Additionally, some prepared feed was also provided daily. Pond fertility was checked every fortnight with Sacchi disk and accordingly steps were taken (Almazan and Boyd, 1978). The fish was reared under these conditions for 4 months (March, 2015 to June, 2015). During rearing optimum oxygen levels in all tanks were maintained by using aerators, pH and temperature were noted daily. Moreover, to avoid strong response towards the handling and netting stress, all fish were inspected daily during the experimental time period. After completion of the rearing period, fish from each experimental group were randomly subjected to behavioural tests.

2.4. Behavioural Study

Before the behavioural tests, fish from each experimental group were randomly selected and housed separately (no. of fry 150; stocking density 1.0 g/L) in 500 L fibreglass holding tanks (three tanks per group). The enriched-reared fish had gravel bed and plastic plants while pond reared fish had pond water in their holding tanks. However, the Barren-reared fish had a bare tank covered in black plastic sheets to mimic the environmental condition in which they had been reared previously. All the groups were fed prepared sinking pellets and provided with 12 hour day/night regime. Each group was held in their new environment for three days before any further studies. All behavioural tests were completed within three days by a group of 3 people and at the end of every day water level of each tank was lowered down to maintain the stocking density

2.5. Boldness Assessment:

Boldness is the propensity of fish to take a risk. It influences the survival, reproduction, and finally the fitness of the animals. Several techniques are, in practice, to measure the boldness in fish like exploratory behaviour and anti-predator behaviour tests (Wilson and Godin, 2009), time taken to emerge into novel environment and propensity of fish to inspect a novel object (Brown et al., 2007).Some researchers conducted boldness tests in familiar environments (Re'ale et al., 2007), while others using novel object and environment for studying anti-predatory and exploratory behaviour (Wilson and Godin, 2009). Here we used latency to leave the shelter, and exploratory behaviours, to assess the effect of rearing environment on boldness.

2.6. Latency to leave shelter

One way of assessing the boldness is to measure the time taken by fish to leave the shelter (Brown et al., 2007). The boldness assay we used was the same as reported by Brown et al. (2005), where a single fish was kept in a darkened, enclosed start box already located in a novel test aquaria. Briefly, during an experiment, the fry was transferred via hand netting (6cm × 6cm) to the start box (12cm length × 12cm width × 24cm height) from fibreglass holding tanks. An individual fish was lifted from the water and held in the hand net for 30 seconds, to follow a typical handling time for such trial, and placed into the start box and left for 2 min to settle the fish (a standard time period for such trial). After that period, a door of the start box (12cm width × 24cm height) in the centre of one wall was opened using a fine monofilament and the time noted within which the fry emerged fully from the start box. Fry that emerged earlier from the start box were considered to be bolder. In each trial, approximately twenty repeats for each experimental group was performed one by one with a new fish. If the fish had not left and remain in the start box for 15 min, the test was ended.

2.7. Exploratory behaviour

Exploratory behaviour, i.e. the readiness toapproach a novel shelter was studied by following the method adopted by Ahlbeck and Holliland (2012) for

research on pikeperch (*Sander lucioperca*) behaviour. Three sides of the test aquaria (36cm length × 24cm width × 24cm height) were covered with black plastic sheets, leaving the long end facing the observers uncovered. The bottom of the aquaria was covered with a 1cm gravel bed. The aquarium was divided into three "habitats". On the left-hand side of the test aquaria, plastic plants occupied one quarter of the area of the test aquaria; on the right-hand side of the aquarium a white, non-transparent plastic "start box" (12cm length × 12cm width × 24cm height) occupied one quarter of the aquarium bottom, and the area between the start box and the artificial plastic plants, were kept open. One fish from each group was placed in the start box and allowed to familiarize the environment for 10 min. After this duration, the observer used a thin rope to slowly open the lid of the box from behind the screen. The lid of the start box was remained open until the end of the trial to measure the time spent by fish in the box area, once leave the box and then come back in the box area. The time taken by the fish from opening to leaving the box was recorded. If the fish had not left and remain in the start box for 30 min, the test was ended. If the fish had left the box, its position in the aquarium was recorded after every 30 sec for 10 min. In each trial, approximately twenty repeats for each experimental group was performed one by one with a new fish.

2.8. Predation (Feeding on live prey)

It is a general observation that mahseer feed in a group (schooling) instead of alone (FAO, 2003). For observing the predatory behaviour of advanced fry, reared in three different environments, five fry at a time originating from the same group was housed in a test aquaria (36cm length × 24cm width × 24cm height), provided with earthworm (novel feed for the enriched and barren environment reared fry while there is a maximum chance that fry from the pond environment previously exposed to the earthworms) at a density of 2 earthworm per litre. Three sides of aquaria were covered with black plastic sheets, leaving the long end facing the observers uncovered. The base of the aquarium was covered with a 1 cm gravel bed with a plastic plant (12cm h) filling one quarter of the tank. To enhance feeding motivation and providing almost similar hunger time, fish of each group in different tanks were fasted at two hrs difference for 24 hours before shifting to the test aquarium. The time taken in min to the first attack on prey was recorded for each fry in the group. Ten

trials per rearing group (three trials at a time) were run with five new fry in each trial. The trial ended when all fish attacked prey at least once, or after 120 min. The feeding behaviour of fry (Table 1) was observed by two observers that had two or three focal individuals to monitor the experimental trial to ensure correct assessment of time to first caught prey as previously described by Ahlbeck and Holliland (2012).

Table 1. Behaviour during feeding on live prey (earthworm).

Behaviour	Description
Inspection of prey	Swim toward and observe prey
Picking of prey	Pick up the prey and suddenly leave them and rapidly move away from prey
Feeding on prey	Start eating

2.9 Anti-predator response

In nature, living with predators is an unavoidable aspect of life for almost all fish. Therefore, to gather information through inspection about the current risk and fine-tune their anti-predator responses to variations in predation risk is a prerequisite for the survival of fish. Predator inspection is a natural response that fish are strongly motivated to perform. Here, the anti-predatory behaviour of the fry reared in different environments was observed using two equal size glass aquaria (36cm length × 24cm width × 24cm height) placed next to each other separated by a removable card divider to prevent visibility between the aquaria. One aquarium contained a live, fresh water Spiny eel (*Mastacembelis armatus*) at least ten times the size of the advanced fry of mahseer that was placed in the other aquarium. The two short ends of the test aquarium containing the eel were covered with black plastic sheets, with the long ends facing the observers uncovered. Three sides of the aquarium containing the mahseer were covered, with the long side facing the eel aquarium uncovered. The bottom of the tank was covered with a 1 cm gravel bed. In the left- hand-side corner of the aquarium, a plastic plant offered a possible refuge as it occupied approximately one third of the aquarium. The eel was allowed to acclimatize in the testaquarium10 min before the tests started while mahseer was allowed to acclimatize for 30 min to avoid unusual behaviour due to handling. After 10 min, the divider was carefully removed,

allowing visual contact between test fish and the eel. The behaviour of the mahseer (Table 2) was recorded every 30 sec during 10min. For each of the three rearing groups of mahseer, twenty trials were run, with one new fish in each trial. To minimize any interference due to the possibility of background alarm cue, water of aquaria was changed before initiating new trial.

Table 2. Behaviour observed during anti-predator response trials.

Inactive Behaviour	**Description**
Freeze	Lying motionless on the bottom or freezing mid motion outside of the refuge
Hide	Motionless or low activity within the refuge
Low activity	Slow movement outside of the refuge
Active Behaviour	
Inspection	Swims toward the predator /larger fish
Move away	The moves directionally (decidedly) away from the predator
Skitter	Rapid movements with frequent changes of direction

3. Statistical analysis

The results are expressed as mean ± SEM. All statistical analysis was carried out using lme4 package of R 3.2.5 (R Development Core Team, 2016). Before proceeding to apply ANOVA for comparison of groups, assumption of normality, homogeneity of variances and additivity of the model were checked by *Shapiro*-Wilks, *Levene's* and *Tukey* 1-dF Test respectively. The effects of changing environment on different behavioural responses were analysed by using one-way analysis of variance (ANOVA) with sub sampling plane, i.e. CRD with sub sampling (Hinkelmann and Kempthorne, 1994), followed by post hoc *Tukey's HSD*. Values of $P<0.05$ were considered statistically significant.

4. Results

4.1 Boldness Assessment

4.1.1 Latency to leave shelter

Boldness assessment or time taken by fish to leave the shelter (start box) was significantly different among the mahseer fry previously reared in indoor barren, enriched tanks and outdoor semi natural earthen ponds (n = 20, mean ± SE: 6.2 ± 1.78 min,2.2 ± 0.79 min and 3.9 ± 0.86 min, respectively; one way ANOVA: $F_{2,171}$ = 30.85,$p < 0.001$; Fig. 1). Fish reared in a physical enriched environment emerged out from the start box significantly ($p < 0.001$) sooner than fish reared in the barren tank (n = 20, mean ± SE: 6.2 ± 1.78, 2.2 ± 0.793, Tukey's post hoc:$p < 0.001$). However, non-significant difference was observed between the fish reared in physical enriched tanks and the outdoor pond environment (n = 20, mean ± SE: 2.2 ± 0.79, 3.9 ± 0.86 respectively; Tukey's post hoc: p= 0.28), while clear difference (Tukey's post hoc: $p < 0.001$) was observed among barren and semi-natural pond reared fish in time taken to emerge from the box.

4.1.2 Exploratory behaviour

In exploratory behaviour tests, there was a significant difference in the percentage of time spent by fry of different rearing groups in box area after 10 min settling time (time to familiarize the start box environment) (n = 20, mean ± SEM: 60.33 ± 6.34, 16.6 ± 4.21, 33.3 ± 4.42, One way ANOVA: $F_{2,\ 171}$ = 86.73, $p<0.001$; Fig. 2). After leaving the start box, fry from different rearing environments showed significant variation in the time spent in open (n = 20, mean ± SEM: $F_{2,\ 171}$ = 111.39, $p < 0.001$; Fig. 2), and vegetation area (n = 20, mean ± SEM, One way ANOVA: F_2,171 = 103.19, $p<0.001$; Fig. 2). Barren environment reared fry spent more time in open area compared to fry reared in in physical enrichment (n = 20, mean ± SEM 23.01 ± 5.02, and 12.6 ± 3.34 Tukey's post hoc: $p < 0.001$; Fig.2), while, no considerable difference was observed with semi-natural pond reared fry (Tukey's post hoc: p = 0.9; Fig. 2). Fry reared in a barren environment spent less time in vegetation than fry reared in physically enriched and conventional semi-natural pond

environments (n = 20, mean ± SEM 16.66 ± 2.55, 70.6 ± 4.41, 43.3 ± 5.31, Tukey's post hoc: $p<0.001$; Fig. 2). The frequency of habitat shifts also differed among different rearing groups (n = 20, mean ± SEM; 8.11 ± 1.33, 4.3 ± 0.333, 6.33 ± 0.33, One way ANOVA: $F_{2, 171} = 21.14$ $p < 0.001$; Fig. 2). Fry reared in a physically enriched environment showed more exploratory behaviour than barren reared fish (Tukey's post hoc: $p < 0.001$; Fig.2). However, no significant difference in habitat shift was observed between fry previously reared in physical enriched and semi natural environment (Tukey's post hoc: $p = 0.2$; Fig. 3).

4.2 Feeding on live prey

Ten trials (5 fish each) each were used from barren, physically enriched tank and semi-natural pond in the feeding on live prey test. A significant difference was observed during live prey inspection (swim toward and observed live prey) among different rearing groups n=10, mean ± SEM: 37.5 ± 6.12, 18.75 ± 3.22 and 21.04 ± 5.25 min one way ANOVA:

$F_{2,85} = 42.51$, $p<0.001$; Fig.4). However, non-significant difference was observed between fish reared in physical enrichment and conventional semi natural environments. (18.75 ± 3.22 min, and 21.04 ± 5.25 min, respectively, Tukey's post hoc: $p = 0.80$). The minimum time for live prey inspection was observed for fish reared in physical enrichment (18.76 ± 3.22 min). Moreover, a significant difference was observed in latency to pick live prey between fish reared in barren and physically enriched environment (21.45 ± 2.39 and 63.54 ± 3.22 min, Tukey's post hoc: $p<0.001$; Fig. 4), while fish reared in the indoor physically enriched tank and the outdoor pond environment did not show any significant difference (Tukey's post hoc: $p < 0.06$; Fig. 4). Moreover, significant differences among groups were also observed in time taken by fry to start feeding on live prey. (n = 10, mean ± SEM; 83.54 ± 5.25 min, 25 ± 10.76 min and 37.70 ± 5.41 min, respectively; one way ANOVA: $F_{2,85} = 97.02$, $p < 0.001$; Fig. 4).

4.3 Anti-predatory response

There was a significant difference in freeze behaviour between the barren,

physical enrichment and semi-natural pond reared fry (n = 20, mean ± SEM; 31.66± 3.33 min, 10 ± 2.89 min and 10 ± 2.61 min, respectively; one way ANOVA: $F_{2,171}$ = 195.20, p<0.001; Fig. 5). However, no considerable difference between physical enrichment and semi-natural pond reared fry was observed (Tukey's post hoc: p = 0.96). Hide and Low activity behaviour was also significantly different among the three rearing groups (n = 20, mean ± SEM: 28.33± 3.33 min, 5 ± 0.89 min and 10 ± 1.41 min, respectively, one way ANOVA: $F_{2,171}$ = 128, p < 0.001: n = 20, mean ± SEM; 15 ± 2.89 min, 8.33 ±1.66 min and 11 ± 1.66 min, respectively, one way ANOVA: $F_{2,171}$ = 12.8, p < 0.001; Fig. 5). However, no difference was observed in hide and low activity behaviour between physical enrichment and semi-natural ponds reared fry (Tukey's post hoc: p = 0.06 and p = 0.32; Fig. 5). Moreover, active behaviour of all the rearing groups showed considerable variation (Inspection: n = 20, mean ± SEM: 6.66 ± 1.66 min, 18.33 ± 2.49 min and 15 ± 1.66 min, respectively, one way ANOVA: $F_{2,171}$ = 37.19, p<0.001: Move away, n = 20, mean ± SEM; 8.33 ± 2.11 min, 31.66± 4.41 min and 25 ± 3.33 min, respectively, one way ANOVA: $F_{2,171}$ = 74.00, p < 0.001: Skitter, n = 20, mean ± SEM; 10.55 ± 1.01min, 26.66± 1.5 min and 28.22 ± 3.33 min, respectively, one way ANOVA: $F_{2,171}$ = 50.78, p<0.001; Fig. 5), but fry reared in physical enrichment and conventional semi-natural pond showed almost similar inspection and move away behaviour (Tukey's post hoc: p = 0.86 and p = 0.99, respectively; Fig. 5).

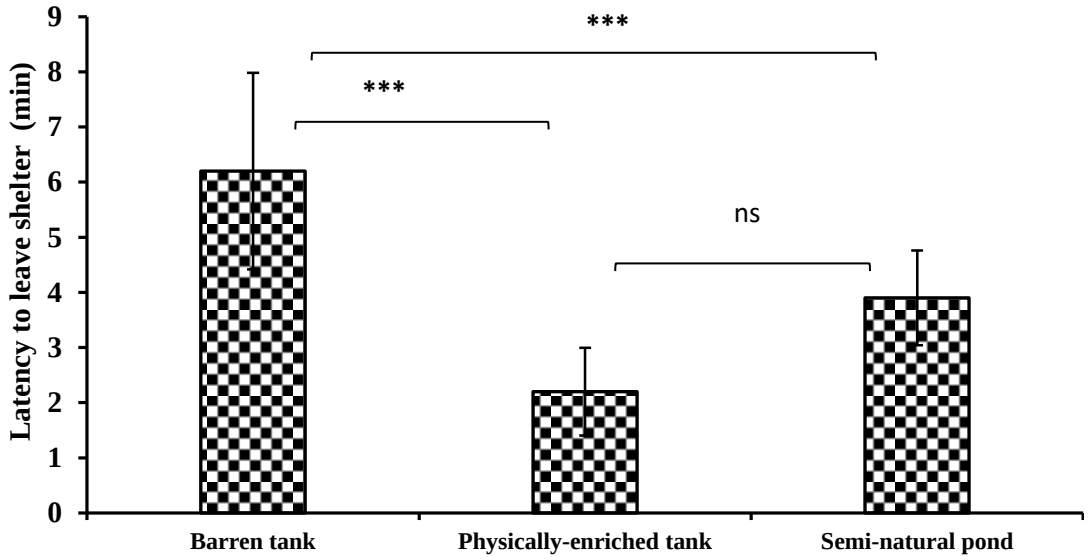


Fig. 1. Latency to leave the shelter (min) by three different rearing groups, barren, enriched and semi-natural environments. Data are mean ± SEM (n = 20). Bar with asterisks differ significantly. * p< 0.05; ** p< 0.01; *** p < 0.001 ns = non-significant.

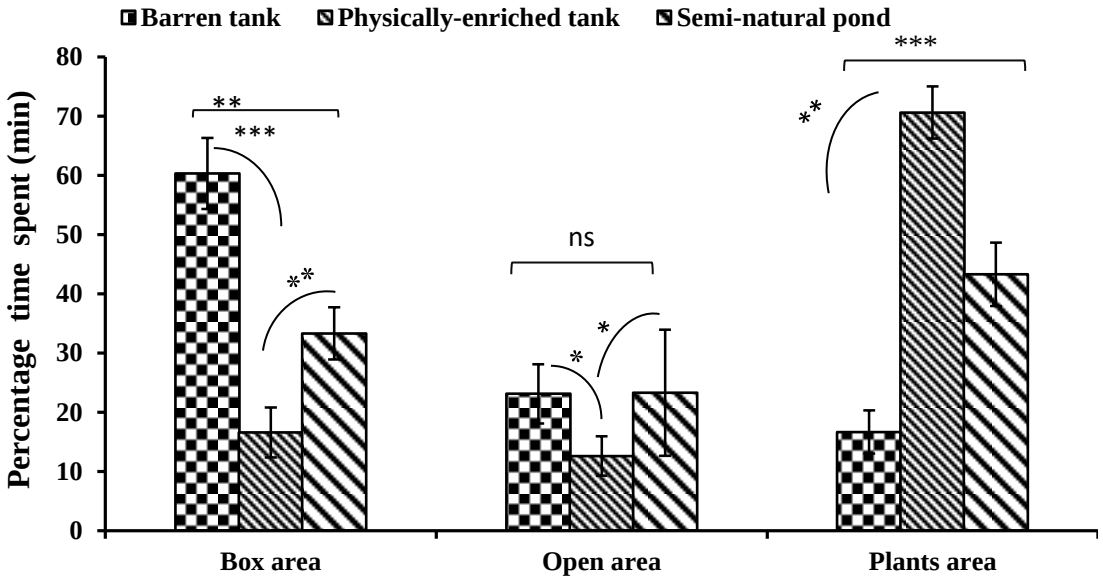


Fig. 2. Percentage time spent (min) in the box, open and vegetation area for three different rearing groups barren, enriched and semi-natural environments. Values are presented as mean ± SEM (n = 20). Bar with asterisks differ significantly. * p< 0.05; ** p< 0.01; *** p < 0.001 ns = non significant.

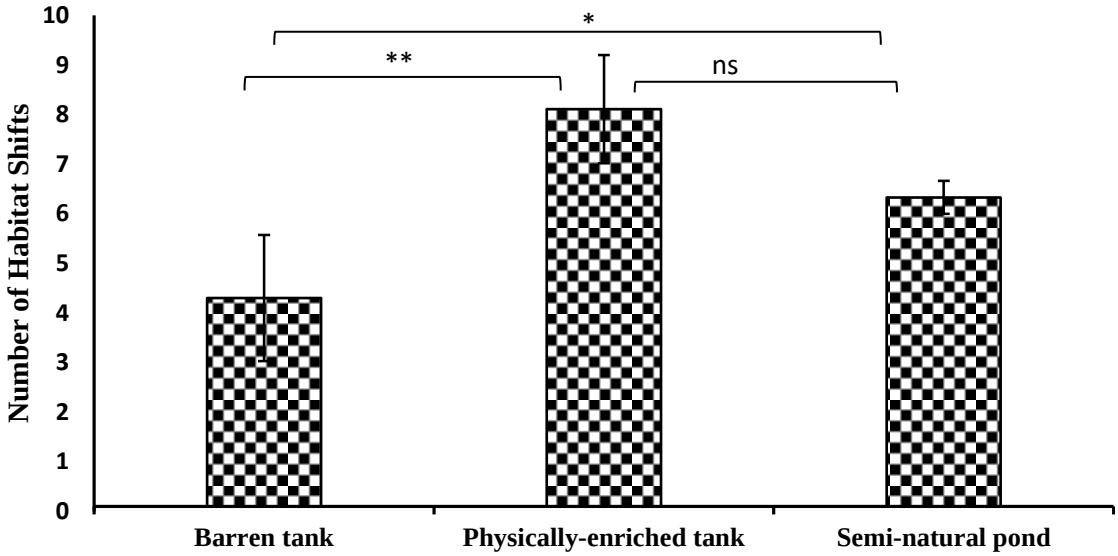


Fig. 3. Number of habitat shifts for three different rearing groups, barren, enriched and semi-natural environments. Data are mean ± SEM (n = 20). Bar with asterisks differ significantly. * p< 0.05; ** p< 0.01; *** p < 0.001 ns = non significant.

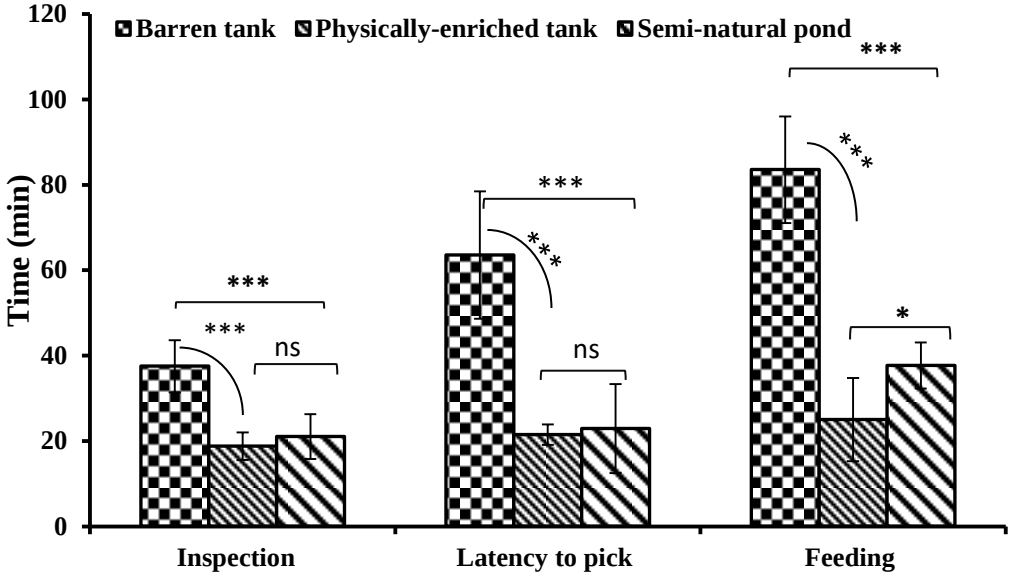


Fig. 4. Time (min) spent performing each behaviour inspection, latency to pick and feeding by fish rearing in three different rearing groups, barren, enriched and semi-natural environments. Values are presented as mean ± SEM (n = 10). Bar with asterisks differ significantly. * p< 0.05; ** p< 0.01; *** p < 0.001 ns = non significant.

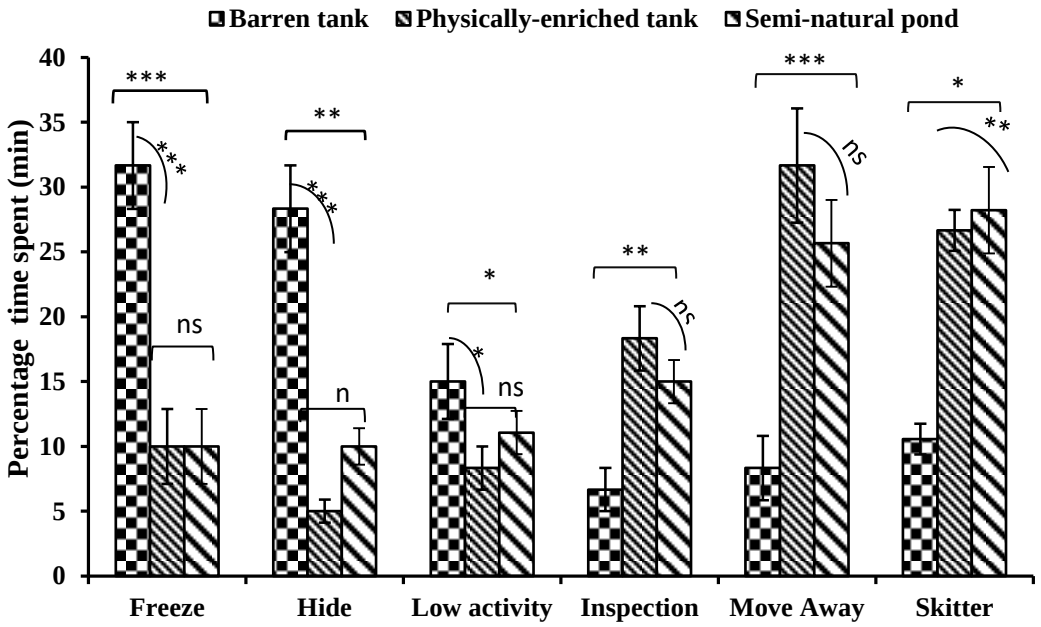


Fig. 5. Percentage of time spent (min) performing each inactive and active behaviour of fish rearing in three different rearing groups, barren, enriched and semi-natural environments. Values are presented as mean ± SEM (n = 20). Bar with asterisks differs significantly. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ ns = non significant.

5. Discussion

In our experiments, clear behavioural differences between groups of fish reared in a barren and enriched environment were observed. The fry reared in enriched tanks and pond environment somehow behave similarly like latency to leave shelter, habitat shift, predation (inspection and latency to pick prey) and anti-predatory behaviour (freeze, hide, low activity, inspection and move away) while both groups behave significantly different compared to the barren-reared group. These findings suggest that the early environment manipulation affects the behaviour of the advanced fry of mahseer. In all trials, enriched-reared fry were more active and bold in their behavioural responses than barren-reared fish, indicating that generally, more active behaviour has developed in the physical enriched environments.

In the latency to leave shelter assay, fish that were previously reared in physical-enriched tank and semi-natural ponds, seemed neophobic to a lesser degree, and emerged earlier from a start box than fish reared in the barren tank environment. This is in accordance with previous studies showing that fish reared in an enriched environment to demonstrate lower levels of neophobia and higher levels of boldness, and than those kept in barren tanks (Sherwin, 2004; Braithwaite and Salvanes, 2005; Fox et al., 2006). Zimmermann et al. (2001) also found that introduction of the newer changes via enrichment by using novel objects and their different arrangements in the rearing environment habituated animals to novelty, thus increasing boldness activity while decreasing neophobia and anxiety. Salvanes and Braithwaite (2005) also reported that, fish exposed to spatial heterogeneity during rearing, display bolder and better curiosity aggravated behaviour. Boldness in fish improved their flexibility and ability to handle with new environments, and prompt rapid resumption of normal behaviour (Brown and Braithwaite, 2004), thus bold fish sooner come out of the stress and enjoy longer foraging durations (Braithwaite and Salvanes, 2005). Kelley et al. (2005) further explained that captive conditions promote the development of the maladaptive risk taking behaviour that increase the chance of predation of reintroduced species in the natural environment. According to Sih et al. (2004) boldness is not always profitable; although at some level it will be adaptive but in another way, bolder individuals may put themselves at more risk.

To acquire information about the surroundings is prerequisite for the survival of animals (Galef and Laland, 2005) and exploratory behaviour make them to collect this information rapidly and more efficiently from the surrounding environment (Braithwaite and Salvanes, 2005). Exploratory behaviour of an organism is affected by the rearing environment (Kellay and Magurran, 2003), In some earlier studies (Braithwaite and Salvanes, 2005; Ahlbeck and Holliland, 2012), fish from enriched environments were faster to enter a new environment which is similar to what was observed in our exploratory behaviour test. In our exploratory behaviour test fish reared in enriched tank appeared to spend more time in the vegetation and less time in an open and box area, however, they are more active and shown higher exploratory response by showing the high number of habitat shift compared to barren reared fish. Camacho-Cervantes (2015) performed exploratory behavioural experiments on guppies and concluded that guppies engaged more suddenly in exploratory behaviour in the presence of vegetation. Moreover, semi-natural reared fish moving around in the aquaria utilizing both the vegetation, start box and open area, while they did not explore the vegetation area at the extent by physically enriched reared fish. It may be due to their previous rearing environment (semi-natural pond) that had a large open space for exploration, but did not have substrate (gravel) and hiding places (PVC pipes). Maximino et al. (2010) previously reported that fish that are less anxious (less shy) are more active (bold) and exploratory. Also anxiety has been shown to decrease in fish through enriched environments (Braithwaite and Salvanes, 2005). Fish that were exposed to rearing heterogeneity in early life were not only bolder, but seek refuge faster than fish previously reared in a barren environment (Salvanes and Braithwaite, 2005).

The barren reared fish in our study, spent more time in the open area and a start box than physically enriched reared fish. However, we cannot conclude that barren fish are more active (bold) and exploratory. Although, according to some scientist, fish spent more time in open area are more active (bold) and exploratory (Sneddon, 2003; Frost et al., 2007), but here it appears that barren reared fish is shy and reluctant to explore the novel environment (vegetation) due to the previous rearing exposure. Moreover, spending more time in the start box after 10 min settling time also indicated its preference to the familiarized environment. Habitat shift results in clearly differentiated exploratory behaviour of barren and enriched environment

reared fish. Millidine et al. (2006) found that Atlantic salmon (*Salmo salar*) behaved less stressed when kept in an environment with access of structural complexity and shelters. Kistler et al. (2011) also reported that zebrafish (*Danio rerio*) and Checker barbs (*Puntius oligoiepis*) give preference to and spend more time in a compartment containing clay pots and vegetation compared to an empty plain compartment without any substrates. Several studies suggest that fish give importance to vegetation and shelters as a source of protection, even if the fish are grown in predator-free environment (Sloman et al., 2011).

Physical enrichment seems to improve flexibility of feeding behaviour (rapid switching to a new food source), foraging and migration (Braithwaite and Salvanes, 2005; Hyvärinen and Rodewald, 2013). Atlantic salmon (*Salmo salar*) reared in variable conditions improved foraging and migration after stocking, compared with barren-reared salmon (Hyvärinen and Rodewald, 2013). As previously reported by Braithwaite and Salvanes (2005), early environmental variability has positive effects on the attraction and consumption of live prey. This is in accordance with our findings as fish reared in an enriched and semi-natural environment started to feed on live prey, significantly faster than barren-reared fish (Fig. 4). Brown et al. (2003) found that only fish with both variable environment and variable food were able to move from one live prey to another despite previous live-prey experience. For hatchery-reared fish, that have been fed non-living food, it can be a challenge to start catching live prey, although studies have shown that such fish can adapt to this quite quickly (Paszkowski and Olla, 1985) indicating that the innate attraction to live prey persists also in hatchery stocks. Even so, there are also studies showing that not all fish from a hatchery succeed in the transition to live prey. Gillen et al. (1981) reported that 11% of the tested individuals of hatchery reared Tiger muskellunge (*Esox masquinongy* × *lucius*), had not switched from pellet to live food in 14 days indicating that all individuals may not adapt to a natural diet after stocking. Still, it might not be the only explanation to the large behavioural difference regarding a new prey (earthworm) as seen in our study. Hunger and stomach fullness are the main factors influencing motivation to eat (Losey, 1995). However, to overcome such factors in our current trial, all of our experimental groups had been starved for 24 hours to increase their feeding motivation. Thus, we assume that the fish were similarly motivated to feed during the trials.

In our current study fry reared in an enriched environment were more prone to perform the active behaviour, including predator inspection. Visual cues often elicit predator-inspection behaviour and the higher proportion of predator inspection in the pond-reared fish may give them a better perception of the risk imposed by an individual approaching. The barren-reared fish had a generally less active anti-predator response, keeping a low profile, predominately remaining motionless. This is in accordance with earlier results reported by Salvanes and Braithwaite (2005) where fish from enriched environments had a stronger anti-predator response than fish from plain environments (Pitcher et al., 1986). Physically enriched-reared fish were more active when exposed to a predator, although there were certain behaviours that did not differ significantly among physical enriched, semi-natural pond environment reared fry, e.g. freeze and low activity behaviour. Like our findings, Salvanes and Braithwaite (2005) also observed that fish from the heterogeneous environment as compared to plane or barren environment use shelter more often. Similarly, Howell (1994) found that anti-predator responses in hatchery-reared are weaker than wild reared fish and evidence from comparison of wild and hatchery juveniles European sea bass (*Dicentrarchus labrax*) of the same size illustrate that wild fish had a stronger anti-predator response, than hatchery reared (Malavasi et al., 2004). This may be due to the dynamics of the rearing environment with wild fish, previously exposed to more variable and complex environment than the hatchery reared fish. Roberts et al. (2011) determine that two weeks of rearing of salmon in an enriched environment considerably reduced risk taking behaviour towards a predator and took a considerably longer time to leave a shelter. The current findings are consistent with those observations from a number of previous studies that antipredator responses are weaker in hatchery reared fish (Howell, 1994; Brown and Day, 2002; Malavasi et al., 2004; Salvanes and Braithwaite, 2005).

5. Conclusions

The projects related to environmental rearing enrichment and pre-release conditioning have been carried out mostly on mammals and birds (Seddon et al., 2007; Griffin et al., 2001), However, our results and those of others previous studies (Braithwaite and Salvanes, 2005; Roberts et al., 2011; Ahlbeck and Holliland, 2012; Näslund, 2013) suggest that fish can probably benefit from manipulation (enrichment)

of rearing enrichment. In the current study, certain behaviours of mahseer were modified through manipulation of physical enrichment in the early rearing environment. These results have important implications for a possible way of improving the outcomes of restocking program of endangered fish species by modifying conventional hatchery-rearing environments. It is suggested that by physical enrichment, the post survivorship of the hatchery reared mahseer can be improved.

GENERAL DISCUSSION

The overall results of the present study indicated the impact of rearing environment in modification of behaviour and stress sensitivity of *T. putitora*. Many factors are influencing the physiology and behaviour of fish, including genetics, environment and the interaction between gene and environment. In such type of studies, variation among populations, species, strains and even within strains often appears (Barton, 2002), therefore experimental conditions and experimental protocols play significant role in the validity of the study. Although, physiological responses are genetically based but alter / modify by individual experiences (Heath et al., 1993; Overli et al., 2005). Here both invasive and non-invasive methodologies were adopted in order to validate our results, get a clear picture and to avoid the factors confound the results.

Free living fish (wild) were collected from River Haro, a breeding site of *T. putitora* while captive reared counterpart of forth generations was purchased from Mahseer Fish Hatchery Hattian, Attock, Punjab, Pakistan, where it was successfully artificially propagated since 2001. The wild caught fish were about 5% more in weight compared to captive reared counterparts, therefore, for physiological stress response study, we adjusted this difference by fixing the stocking density instead the number of fish. It is reported that small variations in body size does not result in considerable changes in metabolism and production of cortisol (Bender et al., 2008). Behaviour is function of generations in captivity (McPhee, 2003) and difference even in a single generation, resulted large changes in behaviour (Álvarez and Nicieza, 2003; Salonen and Peuhkuri, 2006), therefore caution was made for obtaining only fourth generation hatchlings for rearing in barren, semi-natural and physically enriched environment.

In summary, captive reared population of mahseer showed atypical stress response i.e., lower sensitivity to stress or low activation of HPI axis and brain monoamenergic (serotonergic and dopaminergic) systems and delay recovery period as compared to wild counterpart (Table, 8, 10, 12, 14, Fig 4 to 9. Similar attenuated response of captive reared fish has already been reported (Winberg et al., 2001; Barton, 2002; Perreault et al., 2003; Larson et al., 2003; Lepage et al., 2005; Iwama et al., 2006; Zuberi et al., 2011; Gesto et al., 2013) and suggested the role of rearing environment in modification of the sensitivity to stress. Like our observations, rapid response and early recovery to stressor of wild or free living populations has already been

reported (Barton, 2002; Iwama et al., 2006; Zuberi et al., 2011). We are confident here that the observed differences in stress response between populations are the result of altered developmental patterns of captive-reared population because the experiment was conducted in replicate of three under similar conditions and both populations were exposed similar stressor. The release of cortisol is temporary and it provides energy to cope stress challenges. However, the prolong released of cortisol by captive reared fish in response to acute stress indicate the adverse effects hatchery rearing on the physiological stress response.

Both wild caught and captive reared fish were large enough to collect blood for the estimation of cortisol, therefore plasma cortisol was determined for evaluation of stress response. However, blood sampling inherent several problems like capturing / removal of fish from group without causing stress to other members as well serial or time dependants sampling (Laidley and Leatherland, 1988), and raise questions to the validity of the stress response study. However, in the present study, we avoided this limitation by dividing experiment groups of both populations in time based series and at a particular time, all fish in a designated aquarium were anaesthetized by the addition of MS222 and sampling was done without causing stress.

For validation of plasma results, the stress response experiment was also conducted in a flow through system and water-borne cortisol was determined. Like us, many fish researchers also adopted both invasive (from blood) and non invasive (through water) techniques for examining the difference in corticosteroids response to stressors (Ellis, 2004; Barton, 2002). Both experimental approaches provide similar typical response in wild caught and atypical in captive reared mahseer and confirmed the negative effect of hatchery rearing environment on the physiological stress responses (Fevolden et al., 1991; Fevolden and Roed, 1993; Pottinger et al., 1994; Fevolden et al, 1999).

The difference in magnitude of post stress, blood glucose level and recovery to normal level among populations (Table 5, Fig. 3), also support the results of cortisol and confirm that both populations are differ in their sensitivity to stress. The elevated levels of glucose indicate the demand of energy rises to cope with the stressful situation (Martínez-Porchas et al., 2009). However, glucose profile in both populations showed an initial fast increase in less than 0.5 hr, most probably by an increase glycogenolytic process in liver in response to elevated blood

catecholamines (Guesto et al., 2016) and a second burst between 45 min and 2 hrs to 4hrs, could be related to the cortisol promoting gluconeogenesis (Mommsen et al., 1999). Similar differences in building peak levels and returning to basal level as compared to cortisol have already been reported (Pickering et al., 1982; Pottinger, 1998; Flodmark et al., 2002).

The concentration of blood circulating cortisol is the indicative of personality trait i.e., boldness. Several studies on fish and other taxa reported the links between stress reactivity and behavioural responses (Øverli et al., 2007) and between boldness traits and stress responses (Carere and van Oers, 2006). Huntingford et al. (2010) reported the link between activation of cortisol receptor gene to coping strategies in carp *Cyprinus carpio* by observing significantly lower activation of cortisol receptor gene in bold fish than in shy fish.

Bold fish had a significantly lower circulating level of cortisol compared to the shy one (Huntingford et al., 2010; Raoult et al., 2011). In the present study, the significantly low magnitude of plasma cortisol after exposure to stress (Table 4, Fig. 2), and more time spend in open areas (Fig. 2, Chp. 2). In behavioural test specify the bold behaviour of captive reared *T. putitora* compared to wild counterpart. Results are in agreement with Raoult et al. (2011) observing the juvenile similar relationship of cortisol between bold and shy *Argyosomus japonicus.* Captive environment promotes boldness in fish (Kelley et al., 2005; Huntingford, 2004) may be due to high density rearing environments and development of trait to compete resources. Moreover, higher energy intake demand and elevated metabolism of captive-reared fish compared to free living counterpart, also force hatchery reared fish to take risk for food and compete others (Lepage et al., 2000; Sundstrom et al., 2004; Killen et al., 2011). The more rapid emergence of captive reared mahseer from shelter indicates curiosity and more risk sensitive behaviours (Einum and Fleming, 2001; Huntingford, 2004) and suggest the negative impact of captive rearing on live skill activity because such conspicuousness makes them more prone to predation in the wild (Kelley et al., 2005). The boldness and reduced stress response of hatchery reared mahseer may explain the low post release survival in wild. Such traits are beneficial for fish destined for the table, but detrimental in conservation strategies.

Brain serotonergic system influences the HPI axis and regulate stress response and behaviour of fish (Winberg et al., 1997; Overli et al., 1999). The elevated 5-HIAA/5-HT and DOPAC/DA ratios in several brain areas and up-regulation of 5-HT synthesis under stress suggested its role in coping stress mechanism (Lepage et al., 2000). Generally, 5-HT stimulation inhabits the active behavioural responses like locomotion, feeding and aggressive behaviour (Winberg et al., 1993; Leibowitz and Alexander, 1998; Øverli et al., 1998). Here like cortisol , the reduced levels of all monoamines in captive reared mahseer in contrast to wild counterpart (Table 8,10, 12, 14, Fig 4, 7 chp.1) also support the view of the development of more curiously, risk taking behaviour in hatchery reared fish. The lower post-stress brain 5-HIAA/5-HT and DOPAC/DA in domesticated fish were well documented (Lepage et al., 2000).

Captive reared and wild caught mahseer, although showed statistically different physiological stress response with respect to blood and water-borne cortisol, blood glucose, 5HIAA/5-HT and NE but except NE all parameters did not show highly significant interactions between population and treatment. It may be due to interbreeding of wild and captive reared fish because of restocking program or due to the small genetic distance between population as we have used 4th generation captive reared fish. Heritability as well as modulation of the stress response by differential exposure to stressor during ontogeny is well documented (Fevolden et al., 1999; Brown et al., 2005; Kelley and Brown, 2010).

Early rearing environment affect the physiology and shaping the behaviour of fish, while enrichment in rearing environment, improve the behaviour and stress sensitivity of fish by modulating HPI axis and influencing dopaminergic and serotonergic systems. Beneficial effects of enriched rearing environment include better food utilization, improved growth and fillet quality, increased healing erosions and survival (Arndt et al., 2001; Coulibaly et al., 2007). In the study, presence of substrates (PVC pipes, stones and vegetation) in the rearing environment act as means of environmental enrichment for *T. putitora* and improve the sensitivity to stress and overall predatory, anti-predatory and foraging behaviour of fish.

Significantly low magnitude of almost all stress parameters in the physical enriched group of fish after exposure to stress as compared to barren and semi-natural groups showed a certain positive effect of environmental enrichment on HPI axis and brain dopaminergic and

serotonergic systems. Fish from barren and semi natural environment reared groups had somewhat higher levels of all studied stress parameters (whole body and water borne cortisol, blood glucose and brain monoamines) as compared to physically enriched reared group. These differences in basal levels may be due to the presence of unpredictable stressors in the rearing environments of barren and semi natural reared groups (Ladewig, 2000). However, overall slightly low basal levels of stress hormones in physically enriched groups (Table 4, Fig. 2, Chp. 3), could be due to the presence of shelter or hiding place. It seems that structural enrichment regulates the external disturbance and lower conspecific aggression (Batzina et al., 2012, 2014).

The somewhat higher magnitude of cortisol, glucose and brain monoamines in the barren reared group of fish (Fig. 1 to 8 chp 3) reflect their physiological stress response comparable to wild one, but sustained high levels of all stress related parameters over time as compared to basal values and prolong recovery from stress suggested their atypical stress response comparable to captive reared fish. The significantly higher basal level of blood glucose in a barren reared group of fish reflects the presence of intermittent stressors in the rearing environment. It seems that in structure-less environment fish don't have any place to avoid external disturbance while low level in the physically enriched group might be related to reduce stress because of the presence of substrate and shelter (hiding place). Similar reduced stress shown by gilthead sea bream (*Sparus aurata)* in the presence of the substrate has been observed by Batzina et al. (2012, 2014). The reduced activation of whole brain serotonergic and dopaminergic system in the physically enriched group of *T. putitora* also support previous results of beneficial effect of environmental enrichment on sensitivity of stress of fish Crucian carp (*Carassius carassius*), Atlantic salmon (*Salmo salar*), (Höglund et al., 2005), Zebrafish *Danio rerio*, (Millidine et al., 2006) and mammals (mice and rats Brenes et al., 2008; McQuaid et al., 2012).

In mammals, both stimulatory and inhibitory effects of stress on dopaminergic activity have been reported (Höglund et al., 2001; Waters et al., 2005). In the present study, DOPAC/DA ratios increased after stress in all rearing groups and indicated the stimulatory effects, however increased at a lower magnitude in a physically enriched environment reared group compared to

barren reared groups. Our results support the other previously reported observations (Backström et al., 2011; Gesto et al., 2008; Øverli et al., 1999; Weber et al., 2012).

Like physiological stress response, enriched environment reared groups also showed improved behavioural responses and somewhat support our physiological response data. Although semi natural and physically enriched groups did not show any significant difference in latency to leave shelter, habitat shift, predation (inspection and latency to pick prey) and anti-predatory behaviour (freeze, hide, low activity, inspection and move away) but their physiological responses in terms of stress sensitivity indicated by magnitude of whole body cortisol, blood glucose and brain monoamines and recovery from stress clearly showed the difference among populations. However, results both physiological stress responses and behavioural tests clearly indicated the negative impact of physically un-enriched (Barren) environment on the behaviour and physiology of fish.

The early emergence of physical-enriched reared fish from the start box indicated their less neophobic behaviour compared to barren reared fish. Many others also observed similarly lower levels of neophobia and higher levels of boldness in the fish reared in an enriched environment (Sherwin, 2004; Braithwaite and Salvanes, 2005; Fox et al., 2006) and suggested an increase in boldness and decrease in neophobia and anxiety by improving enrichment with novel objects having variation in the arrangements (Zimmermann et al., 2001). Salvanes and Braithwaite (2005) used spatial heterogeneity during rearing of fish and observed bolder and better curiosity aggravated behaviour.

Boldness of physically enriched environment reared *T. putitora* (Fig 1, Chp 4) and their early recovery from stress (Table 2, 4 Fig. 1, 2, Chp. 3), showed their flexibility and ability to handle with new environments, and prompt rapid resumption of normal behaviour (Brown and Braithwaite, 2004). It is reported that bold fish sooner come out of the stress and enjoy longer foraging durations (Braithwaite and Salvanes, 2005). Although, boldness at some level are adopted, but not always profitable (Sih et al., 2004), but here physically enriched environment reared fish showed other improved behaviour (exploratory, anti-predatory and predatory etc.) (Fig 2 to 5 Chp. 4), and indicated their ability to tackle the situation instead of rapid maladaptive risk taking behaviour. It is observed that in exploratory behaviour test, enriched environment

reared fish spend more time in the vegetation and less time in an open and box area, (Fig. 2 Chp. 2). However, they are more active and shown a high number of habitat shift compared to barren reared fish (Fig. 3, Chp. 2) . Similarly, guppies engaged more suddenly in exploratory behaviour in the presence of vegetation (Camacho-Cervantes, 2015). However, semi-natural reared fish did not explore the vegetation area at the extent by physically enriched reared fish. It may be due to their previous rearing environment (semi-natural pond) that had a large open space for exploration, but did not have substrate (gravel) and hiding places (PVC pipes).

Maximino et al. (2010) previously reported that fish that are less anxious (less shy) are more active (bold) and exploratory. Similar is the case here, physically enriched reared *T. putitora* showed low level anxiety indicated by low activation of HPI axis, serotonergic and dopaminergic system and improved exploratory behaviour. Braithwaite and Salvanes (2005) also suggested that fish exposed heterogeneity in early life seek refuge faster than fish previously reared in a barren environment.

Conclusion

In conclusion, the present study has demonstrated that wild-caught mahseer has shown different stress response and life skills behaviour compared to the captive-reared counterpart. These findings suggest that captive rearing environment led mahseer in the physiological and behavioural divergence from wild fish. The physiological stress response, which compared the blood plasma (invasive) and water-borne (non-invasive) cortisol, blood glucose and brain monoamines of wild mahseer with that of their captive-reared mahseer, revealed differences in the activation HPI-axis, dopaminergic and serotonergic system (Stress sensitivity), while behavioural study revealed differences in the frequency of boldness, exploratory and anti-predatory behaviours. Mahseer reared in physically-enriched environment displayed lower post-stress brain 5-HIAA/5-HT, DOPAC/DA and HVA/DA ratios and NE level than reared in barren and semi-natural environment. On the other hand, fish reared in a barren rearing environment more sensitive to stress response indicated by elevated whole-body cortisol, blood glucose, and Ne levels. Our results and those of others previous studies suggest that fish can probably be benefited from the manipulation of rearing environment (enrichment). In the current study,

physical enriched environment reared fish showed improved life skill activities (exploratory, predatory and anti-predatory behaviour) physiological stress responses (low level of anxiety, low magnitude of stress hormones, early recovery from stress). These results have important implications for a possible way of improving the outcomes of restocking program of endangered fish species by modifying conventional hatchery-rearing environments. It is suggested that by physical enrichment, the post survivorship of the hatchery reared mahseer can be improved. Thus the outcomes suggest a modification of the hatchery rearing environment in such a way that it will produce fish, behaviorally comparable to their wild counterpart.

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