Heamatological parameters as first line indicators of fishing stress in different fish species

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By

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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.

Zia Ur Rehman

CERTIFICATE

Certified that the thesis entitled as "Heamatological parameters as first line indicators of fishing stress in different fish species" submitted by ***Zia Ur Rehman*** is accepted in its present form by the Department of Zoology, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan as satisfying the thesis requirement for the degree of Master of Philosophy in Zoology.

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Table of content

List of tables iii

List of figures v

ACKNOWLEDGEMENT vii

Abstract viii

Introduction 1

Aims and objectives 13

Objectives of the current study are: 13

Material and Methods 14

Experimental site and laboratory work 14

Ponds preparations 14

Fish stocking and experimental setup 14

Physicochemical parameters 15

Sampling 15

Hematological indices 15

Histology 16

Blood cell count 16

Technique of differential leucocyte counting 17

Serum cortisol extraction 17

Statistical analysis 18

Results 19

Physiochemical parameters 19

Hematological indices 21

Blood cells Count 42

Histology 48

Serum Cortisol 52
Discussion 55
References 62

List of tables

Sr No	Caption	Page No
Table 01:	Physiochemical parameters of experiment	20
Table 02A:	Hemoglobin in studied species of fish before and after stress	22
Table 02B:	Hematocrits in studied species of fish before and after stress	24
Table 02C:	MCH in studied species of fish before and after stress	26
Table 02D:	MCHC in studied species of fish before and after stress	28
Table 02E:	MCV in studied species of fish before and after stress	30
Table 02F:	MPV in studied species of fish before and after stress	32
Table 02G:	Platelets in studied species of fish before and after stress	34
Table 02H:	RBCs count in studied species of fish before and after stress	36
Table 02I:	RDW-CV count in studied species of fish before and after stress	38
Table 02J:	WBCs count in studied species of fish before and after stress	40

Table 03:	Heterophil cell counts in studied species of fish before and after stress.	43
Table 04:	Lymphocytes cell counts in studied species of fish before and after stress.	44
Table 05:	Monocytes cell counts in studied species of fish before and after stress.	45
Table 06:	Eosinophils cell counts in studied species of fish before and after stress.	46
Table 07:	Basophile cell counts in studied species of fish before and after stress.	47
Table 08:	Serum Cortisol in studied species of fish before and after stress	53

List of figures

Sr No	Caption	Page No
Figure 1	Slide preparations and cell count	17
Figure 2	T. test indicated significant differences of Hemoglobin level in control and stressed condition among the different fish species	23
Figure 3	T. test indicated significant differences of Hematocrits level in control and stressed condition among the different fish species	25
Figure 4	T. test indicated significant differences of MCH level in control and stressed condition among the different fish species	27
Figure 5	T. test indicated significant differences of MCHC level in control and stressed condition among the different fish species	29
Figure 6	T. test indicated significant differences of MCV level in control and stressed condition among the different fish species	31
Figure 7	T. test indicated significant differences of MPV level in control and stressed condition among the different fish species	33

Figure 8	T. test indicated significant differences of Platelets level in control and stressed condition among the different fish species	35
Figure 9	T. test indicated significant differences of RBCs Count level in control and stressed condition among the different fish species	37
Figure 10	T. test indicated significant differences of RDW-CV level in control and stressed condition among the different Fish species	39
Figure 11	T. test indicated significant differences of WBCs Count level in control and stressed condition among the different fish species	41
Figure 12	Slides showing the Serum Histology of fish mori (A) Control (B) after stress.	37
Figure 13	Slides showing the Serum Histology of fish Rohu (A) Control (B) after stress.	38
Figure 14	Slides showing the Serum Histology of fish Silver carp (A) Control (B) After stress.	39
Figure 15	Changes in the post stress concentration (ng/L) serum cortisol in the studied fish species	54

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Abstract

For carp fish species, there is no information available regarding the netting stress response during fish harvesting. It is supposed that physiological stress occurred after the capture of fish through netting. There are different studies which used different methods to access the stress level in fish. We conceptualized the study to evaluate the netting stress response in different species of fish including Rohu, Mori and Silver carp through hematological parameters and cortisol level. Two groups were made one as a control and other was experimental group. After the experiment, the hematological parameters including white blood cells (WBCs/$\times 10^3$µL), hemoglobin (HGB/g/dL), hematocrit (HCT %), mean corpuscular hemoglobin concentration (MCHV/g/dL), hematocrit (%), red blood cells (RBCs/$\times 10^6$µL), mean corpuscular hemoglobin (MCH/ g/dL), mean cell volume (MCV/fL), platelets (/ulRDW-CV (%)), MPV (fl) and platelets (PLTs/×10 6 µL), Blood histology and blood cells count were also calculated in both groups. Apart from this, total Serum cortisol was extracted in both groups to measure the stress level. Our results showed that there was significantly difference in all the parameters studied in our investigation. The findings indicated that netting stress cause the variation in the above-mentioned parameters which are dangerous for fish health. Our findings provided the founding principles of these netting stress in fish and introduces their potential as continuous monitoring tools. Finally, we consider promising avenues of research that could be prioritized in the field of stress physiology of fishes.

Introduction

Basking in the pleasant sun of the dawn of the new millennium, our world has witnessed a paradigm shift in terms of globalization, urbanization and most importantly in human sociological and cultural behavior. Technology has been the single and paramount driving force of civilization attempting to ease out the difficulties of man, with luxuries of increasing comfortable living. Today, the art of lifestyle revolves around machines or automated tools. Modern voice recognition systems have even relieved man from physically operating the machines - the computer performs the task on mere voice commands.

Unfortunately, the position of the Third-World countries is not that impressive. Pakistan is as one such country which does not have that access to technology as the harbingers of the west. The remote villages of the country still runs on bull-carts as compared to the bullet trains of the western counterpart. As an inevitable consequence, labor intensive pofessions are yet to taste the fervor of mechanization and are extensively practiced in the hook and comer of the sub-continent. Fishing in Pakistan is one such profession where manual labor is still indispensable, owing to both technical and economic constraints. Even the nature of the job calls for exaggerated physiological stress.

Fishing has been a very important component of rural economy and an integral part of life and culture of Pakistan since time immemorial. Experts have pointed out the potential of the inland water resources of the country, which can be exploited as fish farming grounds to meet increasing demands for animal protein for a very substantial segment of the population. Presently this enterprise is more income generating owing to

local market forces and growing demand and has been transformed from a mere traditional activity to one based on more improved management practices.

Rohu *(Labeo rohita)*, Mori (*Cirrhinus mrigala*) and Silver carp (*Hypophthalmichthys molitrix*) is extensively dispersed in Pakistan, Bangladesh and India's rivers (Rafique *et al*., 2012). These are valued edible fish, has distinctive marketable and aquaculture prominence throughout its natural distribution range, extending from Indus, Ganges and Brahmaputra river basins (Luhariya *et al*., 2012). They are being extensively farmed all over the country and accounts for pronounced yield (Das *et al*., 2011). They are of the top 10 primarily vital aquaculture species in the world.

Rohu lives in freshwater; brackish; benthopelagic and potamodromous and is widely distributed in Asia: Pakistan, India, Bangladesh, Myanmar and Nepal. Its maximum length is 200 cm and reported age: 10 years. Dorsal fin with 1/12 1/2 radiation fin branches; low profile for sharp head; short dorsal fin with inner rays of branches shorter than head; The anterior scales are 12-16 with a nose without the lateral lobe. Rohu adults live in rivers. They can live in groups as well as tends to live alone. They eat plants. The breeding season is usually accompanied by rainfall in the southwest. Breeding occurs in rivers that are flooded (Jayakumar *et al*., 2018; Khalid *et al*., 2018; Shah *et al*., 2019).

Silver carp lives in clean water; brackish; benthopelagic; potamodromous at 0 - 20 m deep. Distributed in Asia: aborigines in the great Pacific canals in East Asia from the Amur to Xi Jiang, China and Hanoi, Vietnam. Distributed worldwide with aquaculture and control of algal blooms. Several countries report negative environmental impacts after the launch. Their maximum length is 120 cm. They contain dorsal frames (value): 1 - 3; soft dorsal radiation (total): 6-7; skin soaps: 1-3; soft radiation: 10 – 14. No barbels are present

(Pendleton *et al*., 2017; Prechtel *et al*.,2018). The final dorsal ray boundary was not included. Adults from 1.5 cm SL eat only phytoplankton while larvae and young males eat zooplankton. Adults breed in rivers or large veins on shallow rapids with dusty or sandy surface, surface water or surface during floods when the water level rises by 50-120 cm above normal. Reproductive conditions create a high current presence (0.5-1.7 m / s), shifted water, temperatures above 15 ° C (usually 18-26 ° C) and high oxygen concentrations (George *et al*., 2018; Mameri *et al*., 2020).

Reproduces only when conditions change (especially those most sensitive to water levels) and then resume as the water level rises. It has been introduced in many countries where its ability to clean dams and other algae water is more important than its food value. One of three or four species of cyprinids produced worldwide in aquaculture exceeds one million tons per year (Zhang *et al*., 2000; Hintz *et al*., 2017).

Mori is freshwater; brackish; benthopelagic and potamodromous. It is found throughout Asia: from the great rivers of the Indian subcontinent. It has become so widespread in terms of aquaculture that the distribution of natural resources can no longer be determined. Its length is 100.0 cm. It has dorsal captions (value): 0; soft dorsal radiation (total): 12-15; vertebrae: 39. gray body gray; 12-15 background radiation with branches. Adults live in fast-flowing rivers and streams. It can tolerate high levels of salt. Adults almost completely eat vegetables. It eats plankton, but also eats algae. Breeding occurs in the vicinity of a body of water at a depth of 50-100 cm above the sand or clay. A 6 kg female can lay one million eggs (1 mm in diameter). It is widely bred in India but fails to reproduce naturally in lakes, so it breeds. Fishing harvests 40 cm of fish weighing 1000 g and for about three years. The most active fish that grows in lakes but breeds in fast-moving

rivers. The little fingers wish to keep the ponds between July and November (Bhowmick & Bhattacharya, 2014; Mayank *et al*., 2016; Dwivedi *et al*., 2017).

Fish respond to capture and management by immune responses that are emphasized above other higher vertebrates (Skomal & Bernal, 2010; Moustafa *et al*., 2020) and stress responses are divided into three levels in fish: first, second, and higher stress response (Dawood, 2020; Ahmadifar *et al*., 2020). Like teleosts, sharks exhibit a primary and secondary response to stress in their blood chemistry (reviewed by Komkom and Bernal, 2010; Dawood *et al*., 2019). More recently, the manifestations of stress in fish have been identified internally and can be linked to: 1) the size of the fish and its ability to swim with the boat / explosion, 2) the ability to respond to stress, and 3) the ability to minimize the stress (Skomal and Bernal, 2010; Elumalai *et al*., 2020). Authentic data on boat deaths from fisher men and for-profit research show that shark species respond differently to being caught in the same fishing gear (Gilman *et al*., 2008; Morgan and Burgess, 2007; Mandelman and Skomal, 2009; Morgan and Carlson, 2010). The stress level and the definition of stress are characterized by definitions and controversies. It has long history. In general, the stress is a nonspecific of any factors response which are disturbing the state of homeostasis (Elumalai *et al*., 2020; Freitas *et al*., 2020).

However, it is a fact that exact and clear concept of stress has not yet been explained and accepted by physiotherapists, biologists, ethologists, environmentalists, and toxicologists. They explained that the stress is the most diverse process that could be observed in any cell, organs, organ system level or even in whole body (Chadzinska *et al*., 2009; Verburg-van Kemenade *et al*., 2017). After analysis in fish species, it is explained as a system where the dynamic balance of organisms known as homeostasis either is

suppressed or disrupted due to the actions of an internal or external stimulus, which is often described as stress. The oppressor's actions are twofold, as they produce disturbance that either depress or disrupt the homeostatic balance and bring about a systematic and behavioral arrangement that is supposed to be satisfying and flexible, allowing the animal to overcome the threat. When an animal is exposed to chronic stress, the stress response may lose its adjustable and inactive value, which can lead to growth retardation, reproductive failure, and reduced viral resistance (Ferlazzo *et al*., 2003; Hodgkinson *et al*., 2017; Mao *et al*., 2018).

The body's response to stress can be pressurized by one or a group of related, normal, or indirect stresses, and is often seen in response to many types of stress. This reaction usually involves all stages of animal planning and collectively referred to as a combined stress response. The present concept of multicultural response is established on Cannon's pioneer studies on the catecholamines role in responding to animal "fight / flight" threats. The latter has highlighted the ambiguity of the several effective mechanisms underlying the antagonists and the essential part of glucocorticoids in their reaction (Joerink *et al*., 2006; Mosser *et al*., 2008; Rőszer, 2015).

Unless recent findings have shown us that several additional hormones will be implicated during the response of stress. These hormones basically respond the pattern of individual stress factor, the prominent role of CAs and glucocorticoids in this response is still widely known. These hormones are the main messengers of two major pathways through which the brain coordinates the stress response: the hypothalamic-autonomic system-adrenal medulla axis and the hypothalamic-pituitary-adrenal axis (Mantovani *et al*., 2004; Sica *et al*., 2008). In response to stress, the animal tries to create stress by rearranging

its natural functions. This raises the energy redistribution which is an evidence for the increase of both neuroendocrine pathways are also important neuroendocrine mechanisms to regulate the collection and distribution of energy under normal conditions and pressures (Martinez *et al*., 2009; Martinez & Gordon, 2014). Although the conceptual framework is based on mammals, its use of fish is well established (Gerber e*t al*., 2002).

Talking about the stress in fish, it might be induced either by different abiotic factors like change in water temperature, pollution etc. or by biotic interactions like parasitism, predator pray relationship, etc. Human activities are also main factors for causing stress in fish (Ali *et al*., 2020).

A stressful situation, whether something environmental, such as a looming work deadline, or psychological, such as persistent worry about losing a job — can trigger a cascade of stress hormones that produce well-orchestrated physiological changes. A stressful incident can make the heart pound and breathing quicken. Muscles tense and beads of sweat appear. Similar situations are happened in class pices during stressfull condition (Howe *et al*., 2004; Savorelli *et al*., 2017).

Preliminary literature on fish showed that the important phenomenon of responses to fish are like those of terrestrial animals including mammals. They are therefore consistent with the normal spinal pattern (Abou-El-Atta *et al*., 2019; Zemheri-Navruz *et al*., 2020). As will be shown, however, fish stress reactions have many common characteristics in this group. Most or all, of the pressure affects the branchial formation and as a result the hydromineral balance, directly and indirectly on the contaminated fish. Another justification is the good understanding of the integument nervous system.

Sensitive perception of stress is a requirement for relieving stress reactions, in fish and other vertebrates (Cai *et al*., 2020; Adel *et al*., 2020).

Fish react to toxic chemicals and many other stressors at higher concentrations more often than can be seen by land animals. Identifying pressure on fish under the field, plowing in water, or laboratory conditions is difficult. Selye (Cai *et al*., 2020; Adel *et al*., 2020) has already emphasized that there is a continuing response to animals in challenging but compassionate, daily occurrences that can be considered stressful (a small type of stress that can be stimulated, "eustress"), and a very strong reaction to threatening and chronic life that can take lead to infections in animals.

In this continuum it is difficult to define the boundary beyond the non-stressful and stressful situations, or between the dynamic response of a challenging but compassionate throw and the combined response to stress when motivation threatens and disturbs. This is a major reason why stress diagnosis is an undeniable decision, even if it is due to the abundance of behavioral, physical, and structural factors used as indicators of stress (Alsop & Vijayan. 2009; Harper & Wolf. 2009; Adel *et al*., 2020).

Other confusing factors are temporary factors in stress response and many factors that increase or enhance the impact of stressors. There is a significant difference in etiology between severe and chronic stress conditions (von Krogh *et al*., 2010; Ali *et al*., 2020). Many variable influences include variables such as temperature and water, season, age, gender, physical condition, social characteristics, inherited or acquired individual characteristics, and difficulty or variability of species. The importance of changing factors means that the effect of stress depends not only on the severity of the stress but also on the

condition and, most importantly, on how the animals experience it. As simple as it is, however, we follow a common practice and call for stimulant stimuli where they are shown to evoke an integrated response to pressure on fish.

This suggests a sudden or extreme change in the physical environment (temperature, humidity, salt), animal contact (edible creatures, insects, intense local competition, food, or sexual partners), and human disturbances, including waterways (net, management, transport, and overcrowding) and water pollution (low water pH, heavy metals, and organic chemicals). Chemicals can have toxic effects on cell and tissue levels and, in addition to a specific threshold, further increase the stress-response response (Camargo *et al*., 2009; Aluru *et al*., 2010; Letcher *et al*., 2010; Adel *et al*., 2020).

However, toxic chemicals are as important as fish pressure, in addition to terrestrial animals and are therefore included in this review. For the integrated response to pressure in fish, the difference between the primary, secondary, and elevated responses (Kassahn *et al*., 2009). The primary response is reactivation of brain centers, leading to greater release of CAs and corticosteroids, and secondary responses are often described as rapid repetitive actions and effects of these hormones on blood and tissue levels, including increased cardiac output, oxygen uptake, and stimulation of energy substrates and hydromineral balance disturbances. Higher responses increase depending on body size and population: growth inhibition, reproduction, and viral response and reduced ability to withstand subsequent or additional pressures. Following this stage, despite the fact that its intensity, especially in relation to the difference between secondary and secondary response, it is increasingly difficult to combine with the present evidence of flexibility and complexity of

the stress response in fish (Gilman *et al*., 2008; Morgan and Burgess, 2007; Mandelman and Skomal, 2009; Morgan and Carlson, 2010).

Stress response includes different physiological changes including modification in blood structure and insusceptible instruments. These progressions include: osmotic unsettling influences, increment in vigorous substrate fixations (glucose, greasy acids), increment in movement of certain chemicals (lactate dehydrogenase, transaminases, in poisonous pressure too cytochrome P-450 and glutatione increment in pressure protein level (HSP, ubiquitin, metallothionein), and a decline in humoral resistant components (lysozyme, antibodies) , and take-up of electrolytes and water into the cells, joined by an fermentation of plasma, and alkalization of erythrocyte cytoplasm Rapid expansion in erythrocyte number (causing even 25% increment in hematocrit) is an aftereffect of spleen constriction (Caldwell and Hinshaw 1994), what's more, 90% of new cells might be delivered inside a few minutes (Houston *et al*. 1996). Some of the time division of coursing cells might be noticed (particularly in the event of hypoxic stress) (Murad *et al*. 1993). These are versatile changes empowering the life form higher energy creation.

To access the health or stress level in fish there are different tools are being used among them the Hematology is the potential one. Hematological indices are being used in conventional tool in human and veterinary medicine (Blaxhall 1972; Bruno and Munro 1986; Rehulka and Minarik 2007). Among hematological indices, the presence of leukocytes and the shape of it in the bold cells are very important (Campbell and Ellis 2007 Tavares-Dias 2006a). All the stresses may result in significant alteration in such important functions as feeding, reproduction, growth, haematology etc. in fishes.

Among these, hematological studies on fishes have assumed greater significance due to the increasing emphasis on pisciculture and greater awareness of the population of natural freshwater resources. Such studies have generally been used as an effective and sensitive index to monitor physiological and pathological changes in fishes (Kayode and Shamusideen, 2010). Hematological evaluation of fish provides valuable facts concerning the physiological response of fish to changes in the external environment. Study of hematological parameters on one hand help in establishing the health status of fish and on other is the cheapest, trusted and well-known tool to monitor the ambient aquatic environment of the fish. Blood is a sensitive indicator of stress and any physiological dysfunctioing in fish‟s body gets reflected as alterations in its blood constituents. The count of red blood cells is quite a stable index which, like any other organisms, fish also tries to maintain within the limits of certain physiological standards using various physiological mechanisms of compensation.

Hematological variables are also finding considerable usage to determine the effects of different toxic substances and stressors as between the external environment and fish circulatory system, there is no linkage is present (Wendlaar, 1997). As an indicator of stress, blood parameters also help to diagnose and describe the general health condition of fish species following stress conditions (Duthie and Tort, 1985 and Roche and Boge, 1996). Blood cell responses are important indicators of changes in the external or internal environment of organisms.

In fishes, exposure to stress can induce either increase or decrease in hematological levels. These changes may tend to vary depending on the fish species, age and cycle of sexual maturity (Luskova, 1997). In corollary to warm blooded animals,

changes/perturbations in the blood parameters of fish can also be used to determine and confirm the dysfunctioning of various organs or tissues. Thus, haematological analysis can definitely enhance and facilitate fish cultivation through early detection of stress and diseases that could affect production performance (KoriSiakpere et al., 2009). Blood being the medium of intercellular and intracellular transport, comes in contact with various organs and tissues of the body and thus can pose a direct threat to physiological functions of the fish. Xenobiotics (like heavy metals/pesticides) rapidly bind to the blood proteins and thus may induce haematological changes on one hand and histopathological on the other (Tyagi and Srivastava, 2005).

Histopathological biomarkers can be best indicators of effects of various stress level as well as a reflection of the overall health of the entire population of an ecosystem (Jordanoska and Kostoski, 2005). These biomarkers are closely related to other biomarkers of stress since many pollutants have to undergo metabolic activation in order to be able to provoke cellular changes in the affected organisms and hence its histology (Mohamed, 2009).

Effects of Pressure on plasma Cortisol elevated level is majority used indicator in stress response of fish. Habitually, level of cortisol is rapidly increased up to high level within few times. Returning to normal stages takes an hour or more. When stress persists, cortisol levels may remain elevated, even though the reality is below very high levels. Such reactions to cortisol are already studied that in many species of fish. It is described that in different fish species that the level of cortisol increases after the stress via handling, toxic chemicals, natural pollution or any other disturbance like rapid change in temperature

On the other hand, basic levels of 50rig / ml or more have also been reported. These high levels can be explained by classifying test specimens, farming conditions, capture process, or environmental factors (Backström & Winberg, 2017; Burgos-Aceves *et al*., 2019). The binding process can significantly promote plasma cortisol levels, albeit to a lesser extent than plasma CA levels. Especially under field conditions, photographic results are unavoidable and much harder to measure than in the laboratory due to lack of proper controls. To date, the lowest levels of wild fish (<8 rig / ml) have been reported with fish screened and submerged by scuba divers (Wojtaszek *et al*., 2002; Burgos-Aceves *et al*., 2016).

High levels of cortisol have been observed during the last stages of gonadal maturation and during childbirth. Other sources of biodiversity are the endless cycles of time and the annual plasma cortisol cycles shown in many species. Most of the daily reported mountains are in the normal range of depressed fish (Burgos-Aceves *et al*., 2019). Also, annual cycles are reported with very high rates of recurrence during the winter. In migratory salmonids, elevated plasma cortisol levels in spring are associated with smoltification, a pre-migration process in seawater (Adhikari *et al*. 2004; Tamizhazhagan and Pugazhendy, 2015).

Cortisol levels can also vary with diet. In the cultivated chicken salmon, (Fought and Vijayan, 2018). received high levels of cortisol in the period before and shortly after these fish reached the ocean under free living conditions. Only a second elevation, probably the result of preventing fish from entering the sea while being physically prepared to do so, was considered a response to pressure (Schreck & Tort, 2016). Therefore, not all elevated cortisol levels can be recorded in stressors. On the other hand, the absence of clear

standards does not always guarantee the absence of pressures. First, in salmonids, low but constant levels of cortisol levels (from O-5 to 10 rig / ml, i.e., below normal concentrations reported as stress levels) contributed to immune system stress and disease resistance (Koakoski *et al*., 2012). Such subdivisions are often difficult to distinguish from the resting stages. Second, contamination is likely to interfere with the cortisol reaction. Low water levels suppressed salmonid cortisol reactions in treatment and confinement (Das *et al*., 2018).

Aims and objectives

Objectives of the current study are:

- To find out fishing stress in different fish species.
- Hematological indices as indicators of fishing stress.
- To determine the effect of fishing stress on cortisol level in different fish species

Material and Methods

Experimental site and laboratory work

For the purpose to study the stress response of different fish species, an experiment in triplicate was conducted and different fish species were reared in communal ponds at Chanawan Fish hatchery Gujranwala while laboratory work was carried out Animal Physiology Laboratory, Department of Zoology, Quaid-i- Azam University Islamabad.

Ponds preparations

All the experimental ponds at Chanawan Fish Hatchery haveing the average size of 0.1 hectar. Before the start of trial, all of six ponds located close to one another were sun dried. Earthwork including repair of dikes was completed. Calcium carbonate (125kg/ha) and cow dung (333.33kg/ha) was used to treat and fertilize the pond and to enhance the productivity of the ponds. Apart from this, fertilizers including urea, diammonium phosphate (1:3) and cow dung were further added to increase the productivity of the ponds. Sacchi's disc was used to check the productivity of the ponds. Then, all the experimental ponds were partially filled with tube well water. But more water was added later when the already filled water become fertile.

Fish stocking and experimental setup

Six ponds were divided into control (n=03) and experimental (n=03). Three fish species including Mori, Rohu and Silver Carp with average body weight of 1.7±0.43 kg was stocked in each pond in almost equal number i.e 30 of each species with the ratio of (1:1:1) and were acclimatized for 30 days. During the period of acclimatization, the prepared feed having 35%CP were offered to fish (2%/ body weight) twice a day ad libitum.

Physicochemical parameters

During the trial, the Physicochemical parameters were systematically recorded on daily basis. The multiparameter was used to check the physiochemical parameters but the total ammonia was checked weekly, using test kit of ammonia for freshwater (HI3824, ROMANIA).

Sampling

After the period of acclimatization, the harvesting was done. For harvesting purpose fish were starved for 24 hours before harvesting. The harvesting of control and experimental ponds were conducted in different fashions. For control, the ponds were carefully drained, total nine fish of each species were captured very carefully and anesthetized using freshly prepared MS-222 solution. The experimental ponds were partially drained and harvested using drag net of mesh size 1.5 cm.

To study different hematological parameters the blood sample was drawn from each fish of both groups. The sterile syringe of 3 ml was used to take the blood from anal pore bellow the tail of the fish. The blood was stored in lavender top K2 VACUTTE® EDTA tube. After taking the blood of fish, the fish were properly labelled and preserved in 10% neutral formalin for skin histology.

Hematological indices

Blood samples collected were used to analyze the hematological indices. Hematology analyzer (Sysmax XS 800i, Japan) was used for taking the complete blood profile including white blood cells (WBCs/$\times 10^3$µL), hemoglobin (HGB/g/dL), hematocrit (HCT %), mean corpuscular hemoglobin concentration (MCHV/g/dL), hematocrit (%), red blood cells (RBCs/$\times 10^6$µL), mean corpuscular hemoglobin (MCH/ g/dL), mean cell volume (MCV/fL), platelets (/ulRDW-CV (%)), MPV (fl) and platelets (PLTs/×10 6 µL).

Histology

For blood histology field stain method was used. Following procedure was used

1. Placed a drop of blood on a slide and spreaded it over an area of about 1 cm^2.
2. Allowed the film to air dry
3. Flooded the slide in Field's Stain A for 2-3 seconds.
4. Washed the slide with distilled water (Agitating gently)
5. The slide was flooded for 3 seconds in Fields strain
6. Washed with distilled water.
7. Smear on slide was air dried
8. After properly stained and air dried, slides were examined under the light microscope with oil emersion lens.

Blood cell count

Number of erythrocytes, leukocyte, platelets, heterophils, lymphocytes, monocytes, eosinophils, and basophile cells were counted

For this purpose, following procedure was adapted.

1. placed a small drop of blood one side of a pre cleaned slid
2. Placed the distributor 45 ° from the slide and moved it backwards to connect to the drop.
3. The drop quickly spread on the slide distribution line.
4. Then, distributed the film on a fast-paced development.
5. The film was 3-4 cm long.
6. Finally counted all the cell under microscopic hold.

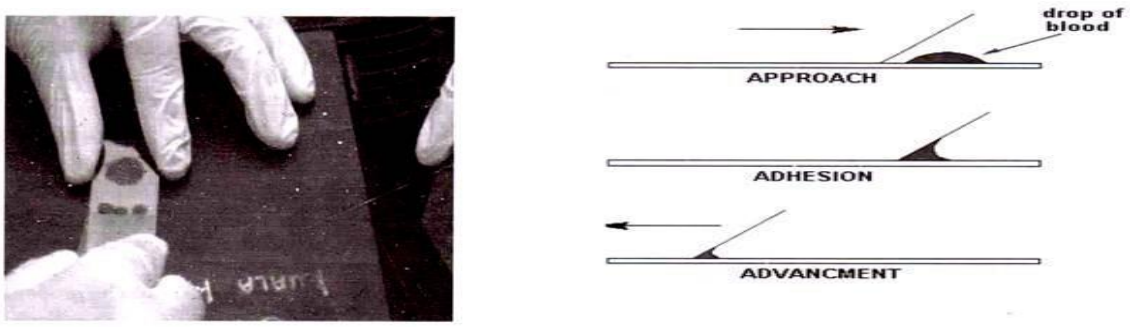


Fig 01: Slide preparations and cell count.

Technique of differential leucocyte counting

Differential leukocyte count (D.L.C) was done in oil immersion, with wide open diaphragm and high up condenser. D.L.C. was done in area where RBC morphology was found good. Unequal distribution of WBC is normal in blood film.

The smaller cells (like small lymphocytes) being in relatively greater numbers in central thicker portions and the larger cells (monocytes eosinophil) being in greater relative number- along the edges and the tail. So, the D.L.C. on a smear depends upon the area of the slide used for counting. To overcome this difficulty both areas were included in counting.

Serum cortisol extraction

To study the stress via cortisol enzyme assay, the total cortisol from serum was extracted. Using the kit DetectX® Cortisol Enzyme Immunoassay (Catalog No K003-H1) and methodology used was based on the procedure describe in Kit manual.

The concentrations of cortisol extracted from serum was authenticated from through ELISA kit by the slopes of the curve created by the serial dilutions. The slopes of curve between observed vs expected values was 0.93, $r^2 = 0.989$ which indicates positive linear relationship. The efficiency of the serum cortisol extraction was calculated by

making serial dilution of standard cortisol to blood. The percent recovery of the methodology (extraction by cartridges and elution by ethyl acetate) was 93.5%.

Statistical analysis

All assays were performed in triplicates and results for each parameter are expressed as mean ± S.E. Before proceeding to apply any statistical analysis for comparison of control and stressed was investigated for normality distribution and variance homogeneity with the help of Bartlett and Shapiro-Wilks test. The effects of harvesting through net on different stress responses were statistically analyzed with T test (T-test type is paired). $P < .001$ was kept the level of significance to find out the statistically difference between control and stressed. GraphPad Prism 5 was used to construct the Graphs.

Results

Physiochemical parameters

The minimum fluctuation was observed in physicochemical parameters which ranged as water temperature (°C) 25.6–28.6, total ammonia <0.5, pH 7.0±0.3 and DO 6.0±0.5 mg/L.

The ponds used for experiment was located near each other and their environmental conditions were also similar. Therefore, in water quality parameters no significant difference was observed. All the data of physiochemical parameters are show in table 01.

Table 01: Physiochemical parameters of experiment

Ponds	**Control**	**Stressed**	**P value**
DO (mg/L)	10.6±2.1	10.1±2.4	0.1483
pH	6.73 ± 0.29	6.32 ± 0.15	0.091
Temperature	26.49 ± 2.4	26.19 ± 1.9	0.212

Hematological indices

Hematological parameters of control and stressed fish of different fish species are presented in Table 02 as well as in figure-2 to figure-11. T-test indicated the highly significant differences in hemoglobin level (Fig 2), hematocrits level (Fig 3), MCH level (Fig 4), MCHC level (Fig 5), MCV (Fig 6), platelets (Fig 8), RBCs level (Fig 9) and WBCs level (Fig 11) between control and stressed fish. While the level of MPV was significantly different between the Rohu and Silver Carp but there was no significantly difference between controlled and stressed fish species of Mori (Fig 7). The level of RDW-CV was not significantly different in Mori and Silver carp fish species but in Rohu the level was significantly different between controlled and stressed (Fig 10).

Table 02A: Hemoglobin in studied species of fish before and after stress.

Fish species	Control	Stressed	P- Value
Mori	11.78±0.29s	9.41±0.26	< 0.0001
Rohu	12.2±0.2	9.28±0.15	< 0.0001
Silver carp	12.13±0.2	8.89±0.3	< 0.0001

a) Hemoglobin

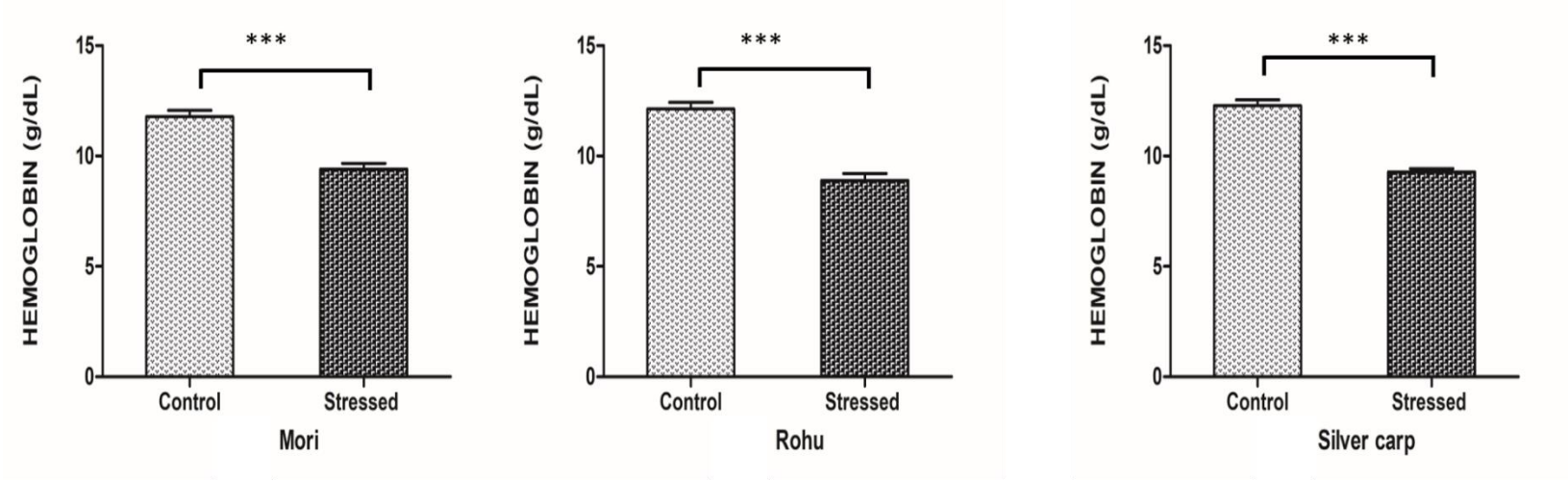


Figure 2: T. test indicated significant differences of Hemoglobin level in control and stressed condition among the different Fish species. The bar shows the values as mean ± S.E (n=9). Starts on the bars showing the level of significance. ($P < .001$).

Table 02B: Hematocrits in studied species of fish before and after stress.

Fish species	Control	Stressed	P- Value
Mori	44.63±0.4	32.6±0.5	< 0.0001
Rohu	46.31±0.7	28.8±0.4	< 0.0001
Silver carp	41.54±1.17	27.82±1.18	< 0.0001

b) Hematocrits

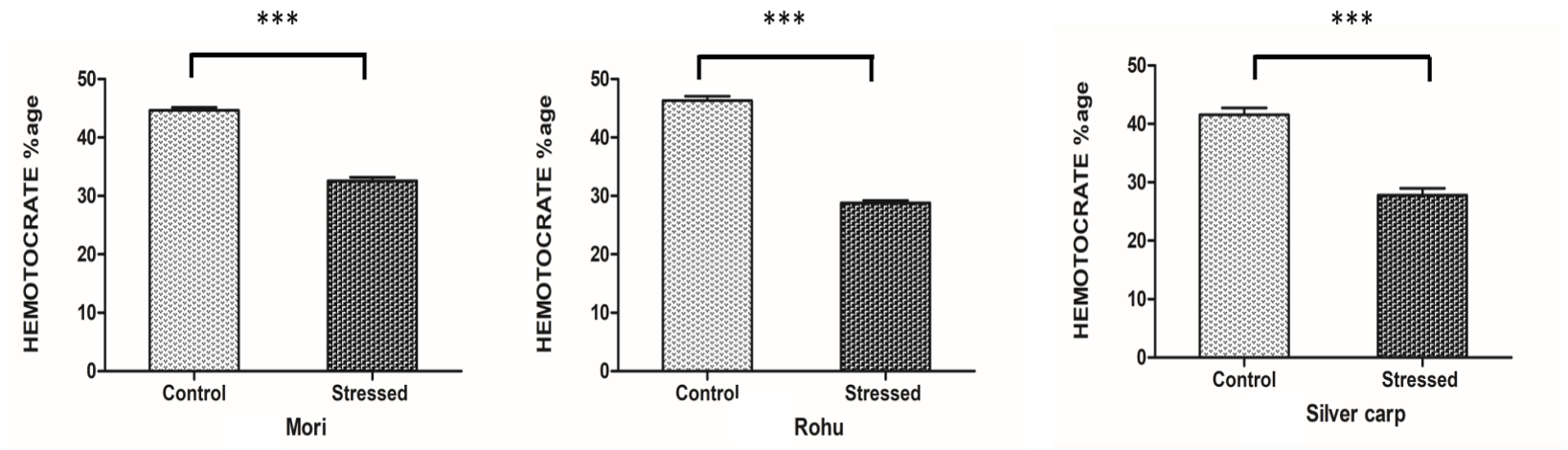


Figure 3: T. test indicated significant differences of hematocrits level in control and stressed condition among the different fish species. The bar shows the values as mean ± S.E (n=9). Starts on the bars showing the level of significance. ($P < .001$).

Table 02C: MCH in studied species of fish before and after stress.

Fish species	Control	Stressed	P- Value
Mori	44.42±0.51	41.4±0.97	0.0146
Rohu	45.03±0.67	32.1±0.76	< 0.0001
Silver carp	40.7±0.78	37.22±1.9	0.0146

c) MCH

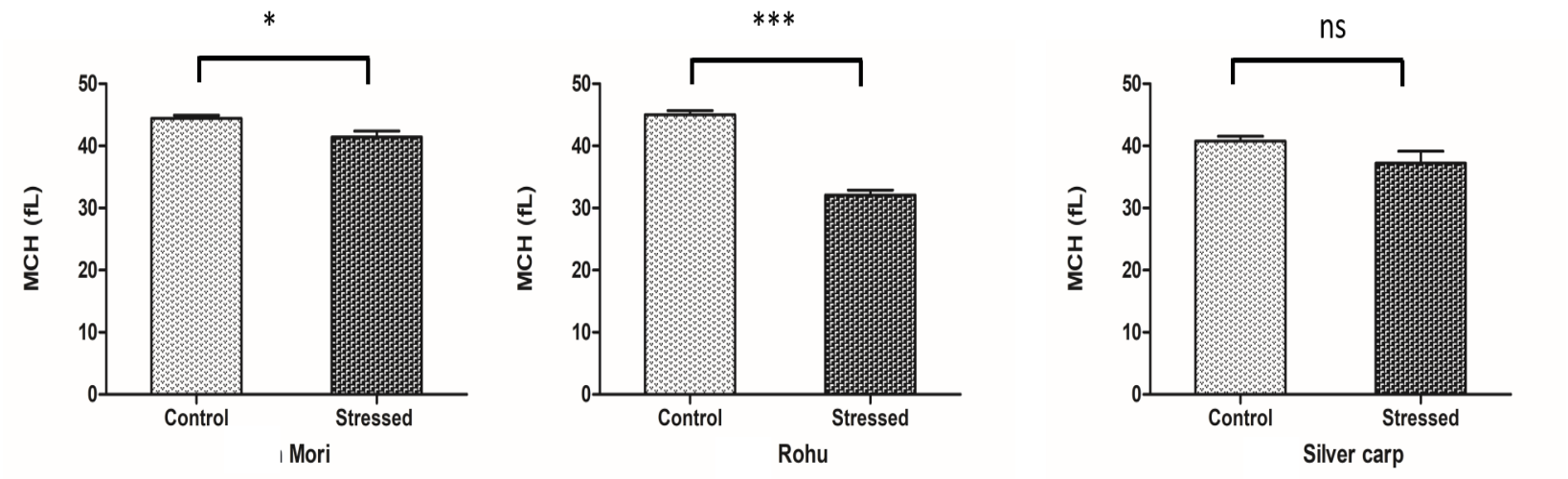


Figure 4: T. test indicated significant differences of MCH level in control and stressed condition among the different fish species. The bar shows the values as mean ± S.E (n=9). Starts on the bars showing the level of significance. ($P < .001$).

Table 02D: MCHC in studied species of fish before and after stress.

Fish species	Control	Stressed	P- Value
Mori	37.37±0.82	32.13±0.39	< 0.0001
Rohu	35.52±1.02	27.6±0.4	< 0.0001
Silver carp	39.16±0.04	34.57±1.7	0.034

d) MCHC

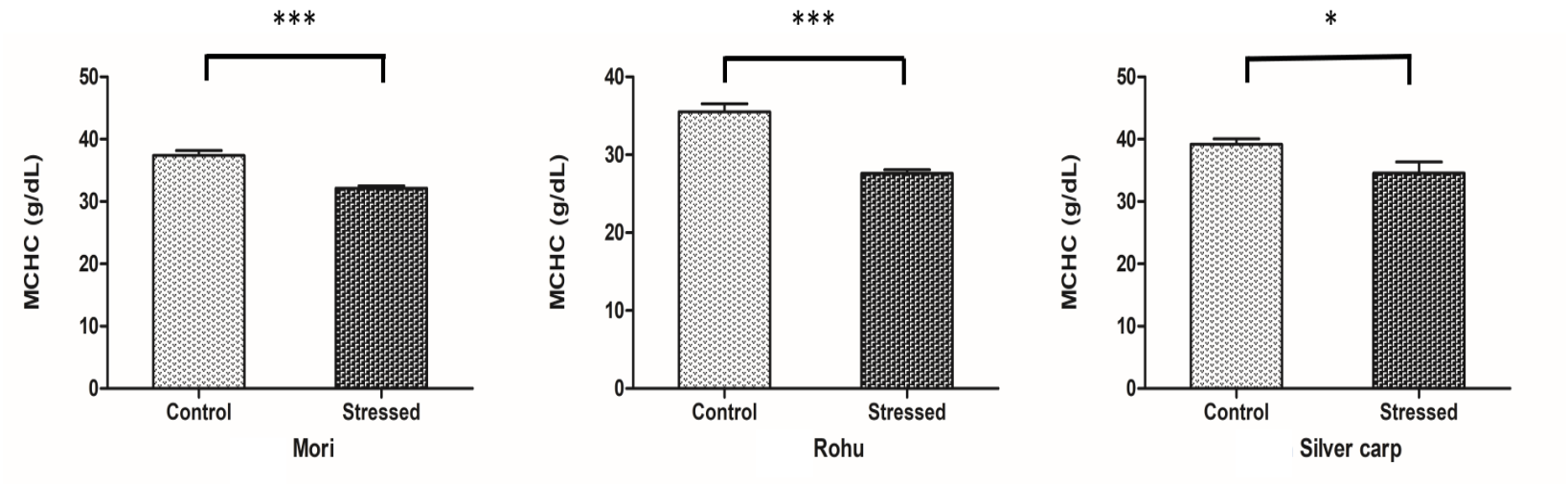


Figure 5: T. test indicated significant differences of MCHC level in control and stressed condition among the different fish species. The bar shows the values as mean ± S.E (n=9). Starts on the bars showing the level of significance. ($P < .001$).

Table 02E: MCV in studied species of fish before and after stress.

Fish species	Control	Stressed	P- Value
Mori	149.26±0.5	140.2±0.95	< 0.0001
Rohu	146.6±1.3	128.43±0.6	< 0.0001
Silver carp	145.51±1.4	130.81±2.2	< 0.0001

e) MCV

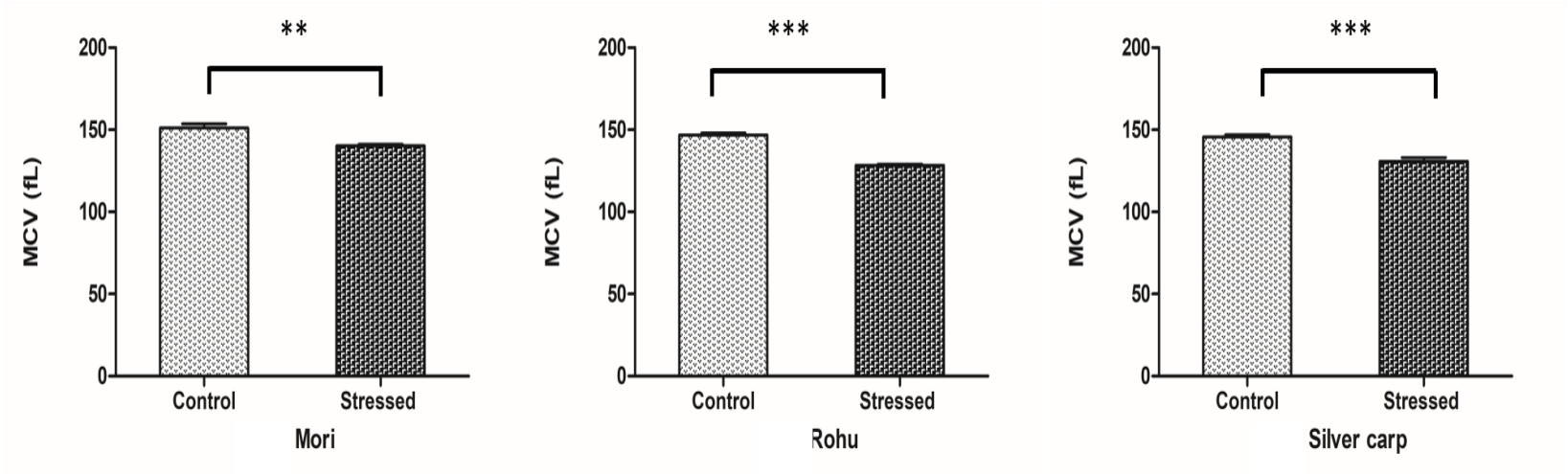


Figure 6: T. test indicated significant differences of MCV level in control and stressed condition among the different fish species. The bar shows the values as mean ± S.E (n=9). Starts on the bars showing the level of significance. ($P < .001$).

Table 02F: MPV in studied species of fish before and after stress.

Fish species	Control	Stressed	P- Value
Mori	8.87±0.3	8.48±0.12	0.208
Rohu	8.925±0.2	8.025±0.24	< 0.0001
Silver carp	9.24±0.3	7.79±0.22	< 0.0001

f) MPV

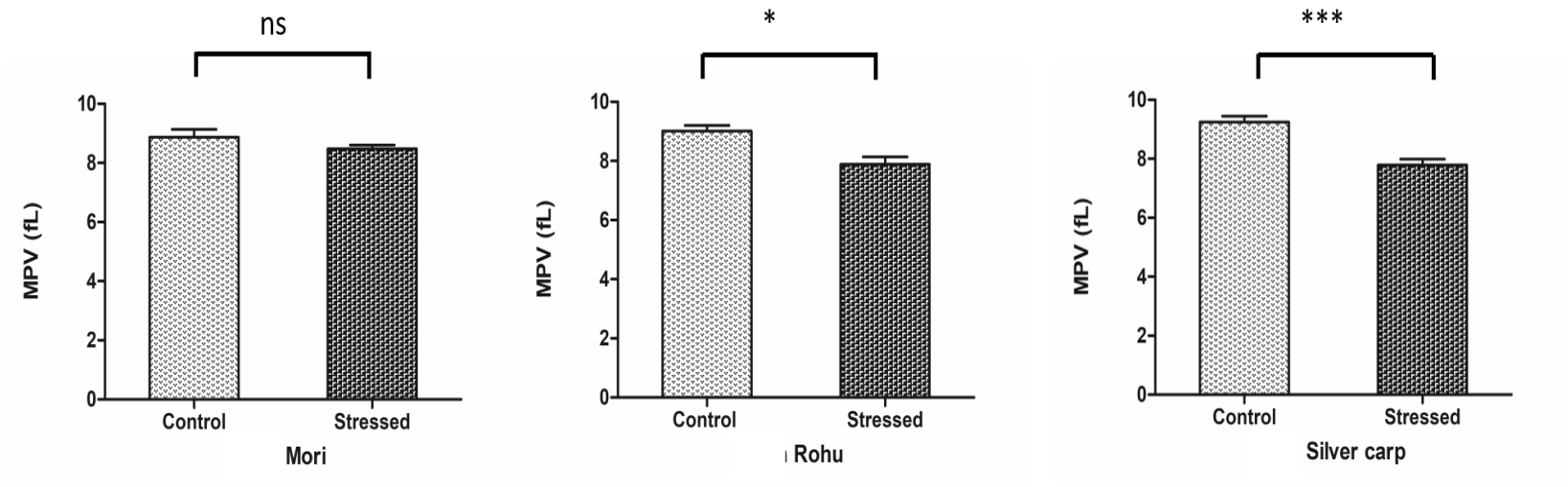


Figure 7: T. test indicated significant differences of MPV level in control and stressed condition among the different fish species. The bar shows the values as mean ± S.E (n=9). Starts on the bars showing the level of significance. ($P < .001$).

Table 02G: Platelets in studied species of fish before and after stress.

Fish species	Control	Stressed	P- Value
Mori	64,222±2228	43,000±1518	0.208
Rohu	75,222±2436	38,556±2242	< 0.0001
Silver carp	55,11±1719	18,778±894	< 0.0001

g) Platelets

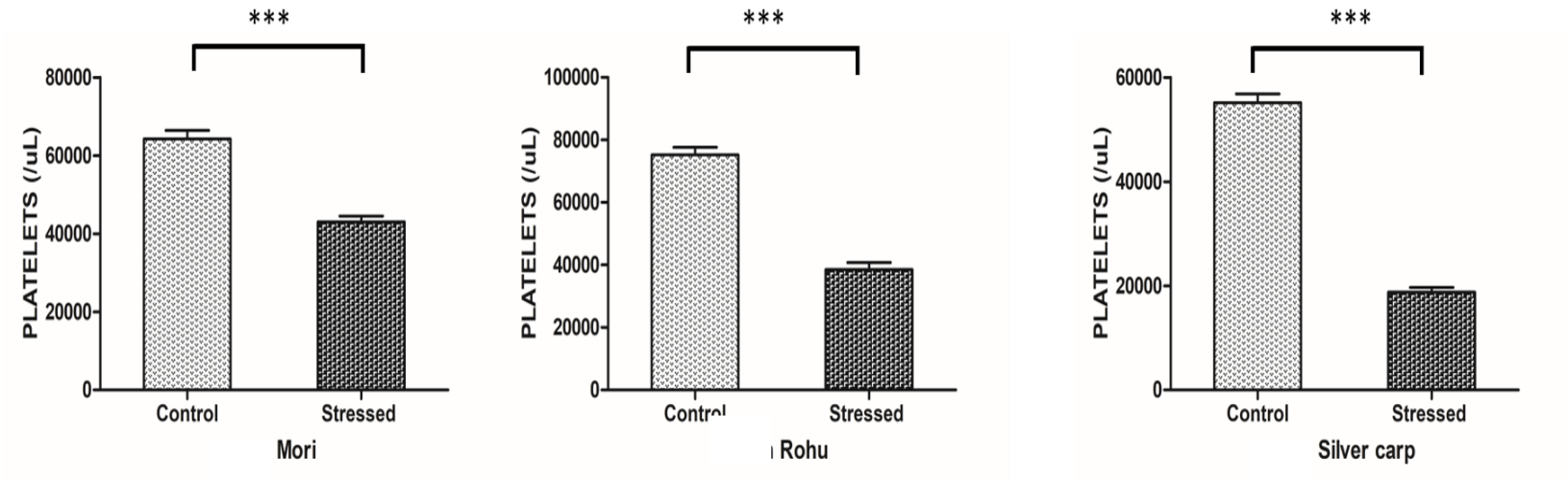


Figure 8: T. test indicated significant differences of platelets level in control and stressed condition among the different fish species. The bar shows the values as mean ± S.E (n=9). Starts on the bars showing the level of significance. ($P < .001$).

Table 02H: RBCs count in studied species of fish before and after stress.

Fish species	Control	Stressed	P- Value
Mori	2.52±0.12	1.87±0.07	< 0.0001
Rohu	2.83±0.24	1.47±0.04	< 0.0001
Silver carp	2.99±0.09	1.92±0.02	< 0.0001

h) RBCs Counts

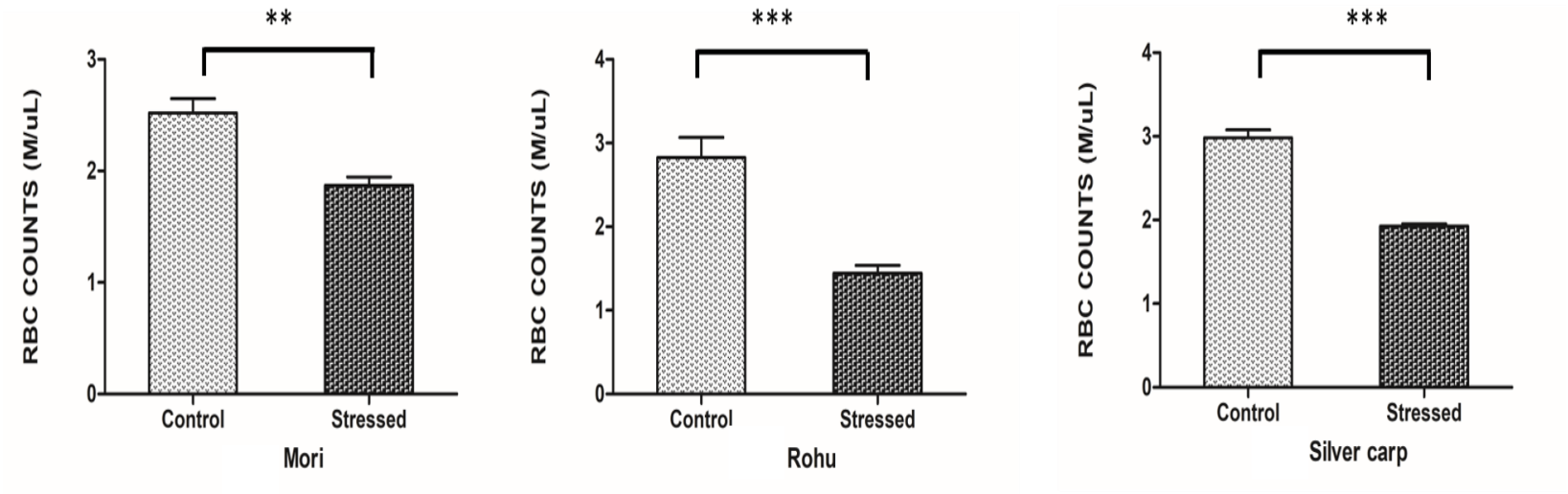


Figure 9: T. test indicated significant differences of RBCs count level in control and stressed condition among the different fish species. The bar shows the values as mean ± S.E (n=9). Starts on the bars showing the level of significance. ($P < .001$).

Table 02I: RDW-CV count in studied species of fish before and after stress.

Fish species	Control	Stressed	P- Value
Mori	22.41±0.77	25.27±0.91	0.029
Rohu	33.08±0.88	20.933±0.36	< 0.0001
Silver carp	20.144±0.85	18.9±1.63	0.514

i) RDW-CV

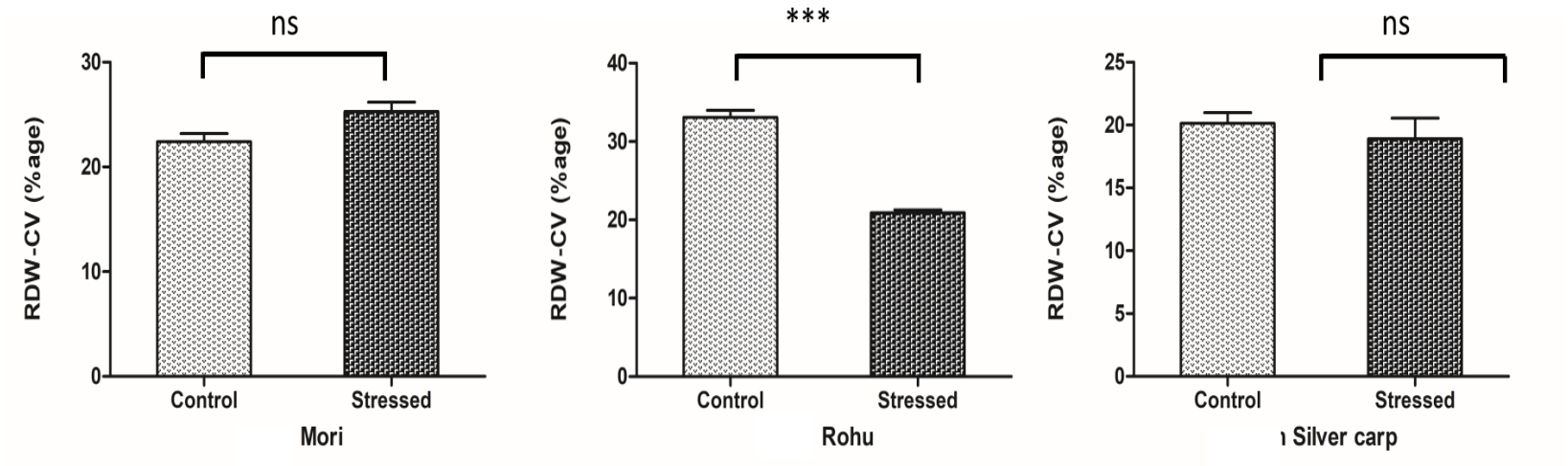


Figure 10: T. test indicated significant differences of RDW-CV level in control and stressed condition among the different fish species. The bar shows the values as mean ± S.E (n=9). Starts on the bars showing the level of significance. ($P < .001$).

Table 02J: WBCs count in studied species of fish before and after stress.

Fish species	Control	Stressed	P- Value
Mori	51422.2±2377	29933.33±1177	0.029
Rohu	39900±2116	23283.33±846	< 0.0001
Silver carp	52866.6±1724	26377.77±1021	0.514

J) WBCs Count

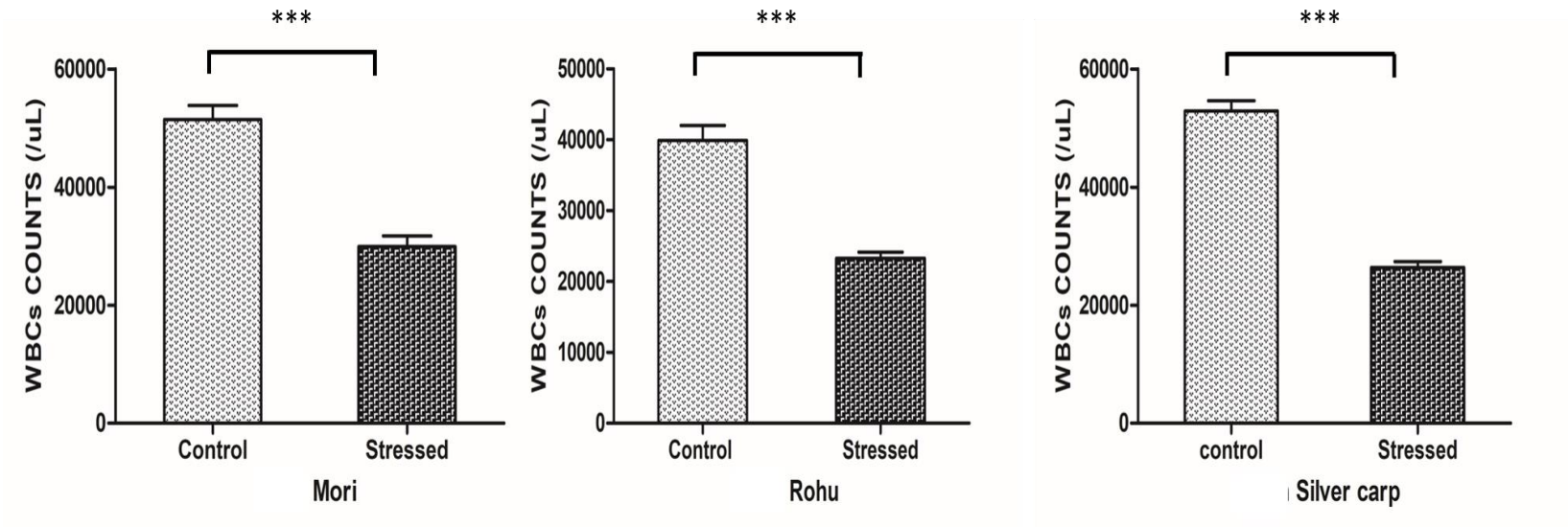


Figure 11: T. test indicated significant differences of WBCs Count level in control and stressed condition among the different fish species. The bar shows the values as mean ± S.E (n=9). Starts on the bars showing the level of significance. ($P < .001$).

Blood cells Count

Blood cell count of control and stressed different fish species are presented in Table 3 to 7. T-test indicated that no significant differences in heterophils (Table 3) lymphocytes (Table 4), eosinophils (Table 6).in Rohu and Silver carp fish species while a significant difference was found between stressed and controlled Mori fish. While the monocytes cell count was not significantly different in Mori but in Rohu and Silver carp the level was significantly different between controlled and stressed (Table 5). The Basophiles cell count was significantly difference in Mori and silver carp but found non-significant in Mori Fish species (Table 7).

Table 03: Heterophil cell counts in studied species of fish before and after stress.

Fish species	Control	Stressed	P- Value
Mori	49.44±0.68	43.44±1.33	0.0064
Rohu	45.77±1.98	42.78±2.537	0.0759
Silver carp	51±1.14	46.22±1.06	0.89

Table 04: Lymphocytes cell counts in studied species of fish before and after stress.

Fish species	Control	Stressed	P- Value
Mori	40.78±0.7	43.11±0.7	0.0068
Rohu	44.44±1.2	48.67±2.6	0.0854
Silver carp	39.22±0.9	40.44±0.3	0.1112

Table 05: Monocytes cell counts in studied species of Fish before and after stress.

Fish species	Control	Stressed	P- Value
Mori	4.78±0.66	6±0.24	0.1547
Rohu	5±0.62	3.89±0.26	0.0304
Silver carp	4.67±0.44	5.89±0.26	0.0023

Table 06: Eosinophils cell counts in studied species of Fish before and after stress.

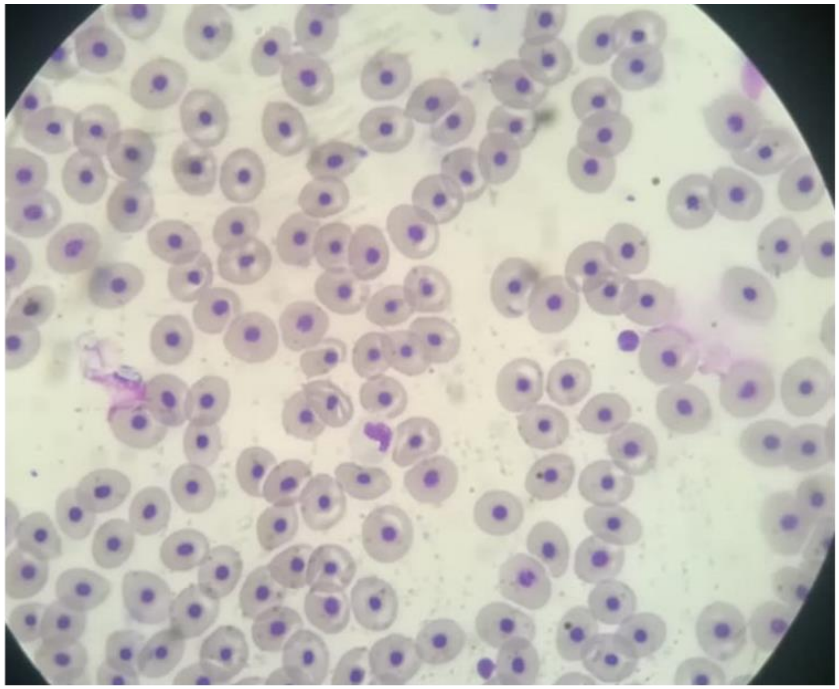
Fish species	Control	Stressed	P- Value
Mori	4.7±0.3	6.7±0.5	0.0053
Rohu	4.9±0.4	4.7±0.4	0.7720
Silver carp	5.3±0.4	6.7±0.6	0.0961

Table 07: Basophile cell counts in studied species of Fish before and after stress.

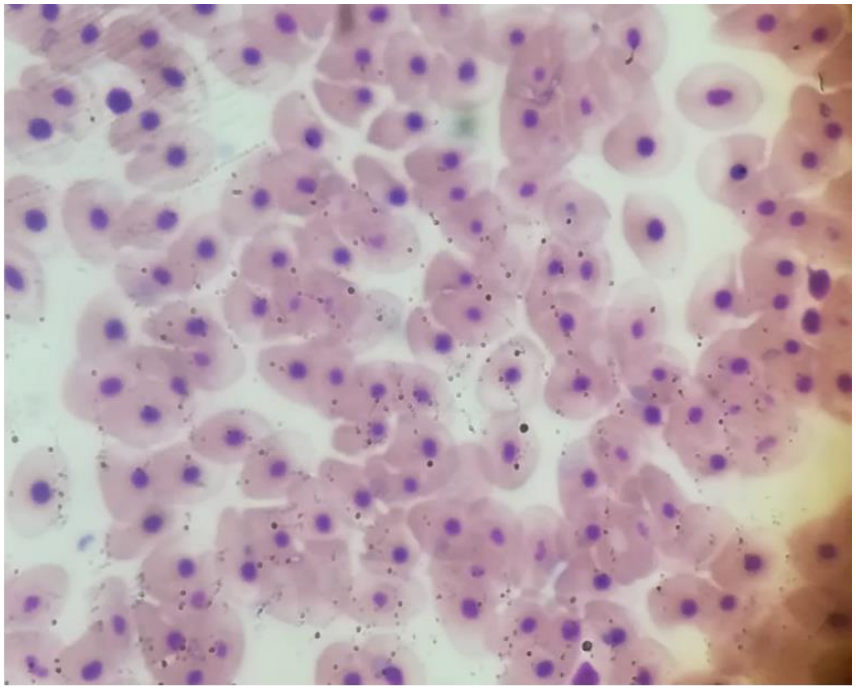
Fish species	Control	Stressed	P- Value
Mori	0.11±0.11	1.22±0.27	0.2083
Rohu	0.78±0.28	0.56±0.16	0.00264
Silver carp	0.33±0.14	1.22±0.16	0.0001

Histology

Blood histology is specialized connective tissue. it consists of cells (erythrocyte, leukocyte, platelets heterophils, lymphocytes, monocytes, eosinophils and basophile. The Figure 12-14 showing the Histology of blood of studied species of fish. In stress fish the lymphocytes of body decreased. Apart from this, WBCs also decrease in stressed fish as compared to control.

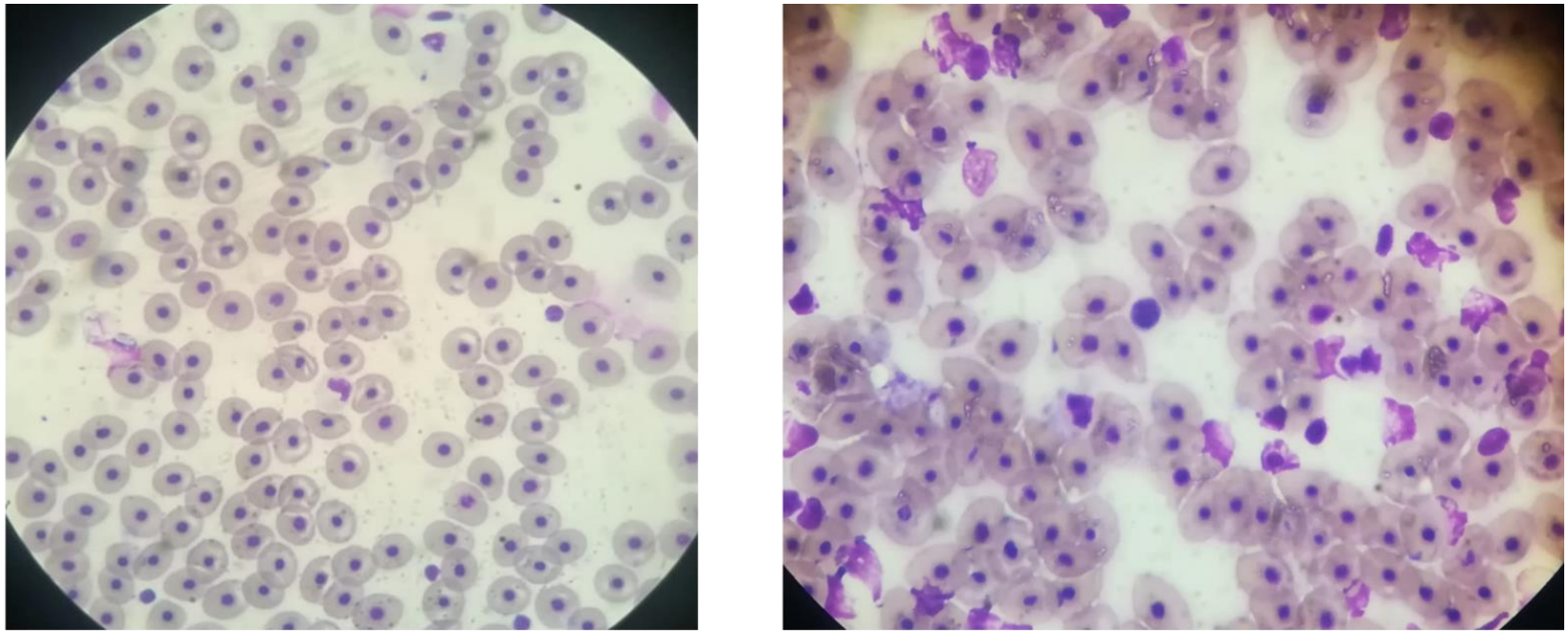


A) Control



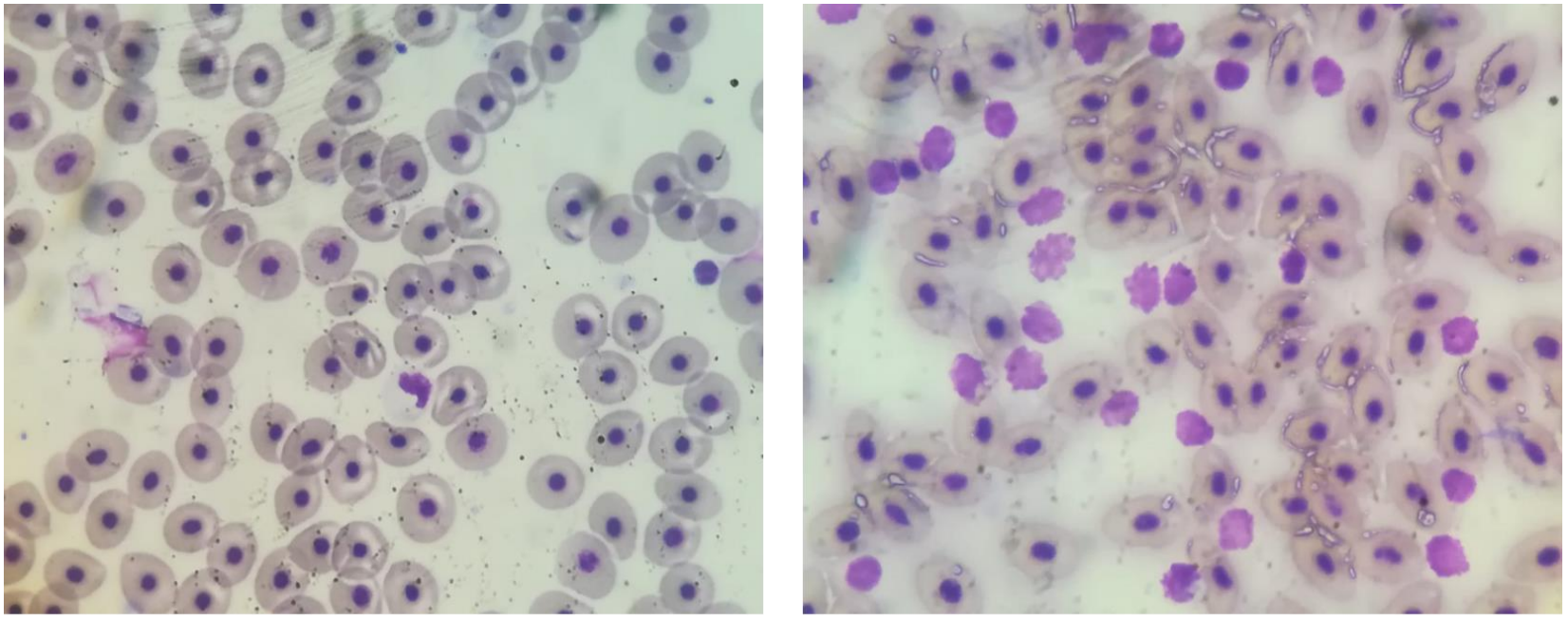
B) Stressed

Figure 12: Slides showing the Serum Histology of fish mori (A) Control (B) After stress.



A) Control B) Stressed

Figure 13: Slides showing the Serum Histology of fish Rohu (A) Control (B) After stress.



A) Control B) Stressed

Figure 14: Slides showing the Serum Histology of fish Silver carp (A) Control (B) After stress.

Serum Cortisol

Serum of control and stress fish species was devoid of cortisol. T test indicated significant differences among the control and stressed in term of cortisol concentration (Table 8 and Fig 15). The pairwise comparison for interaction between the treatments and populations also indicated highly significant effects of confinement treatment in both populations controlled and stressed.

Table 08: Serum Cortisol in studied species of fish before and after stress.

Fish species	Control	Stressed	P- Value
Mori	166.03±5.6	221.02±6.2	< 0.0001
Rohu	154.40±5.2	241.59±5.2	< 0.0001
Silver carp	138.28±4.9	217.717±7.5	< 0.0001

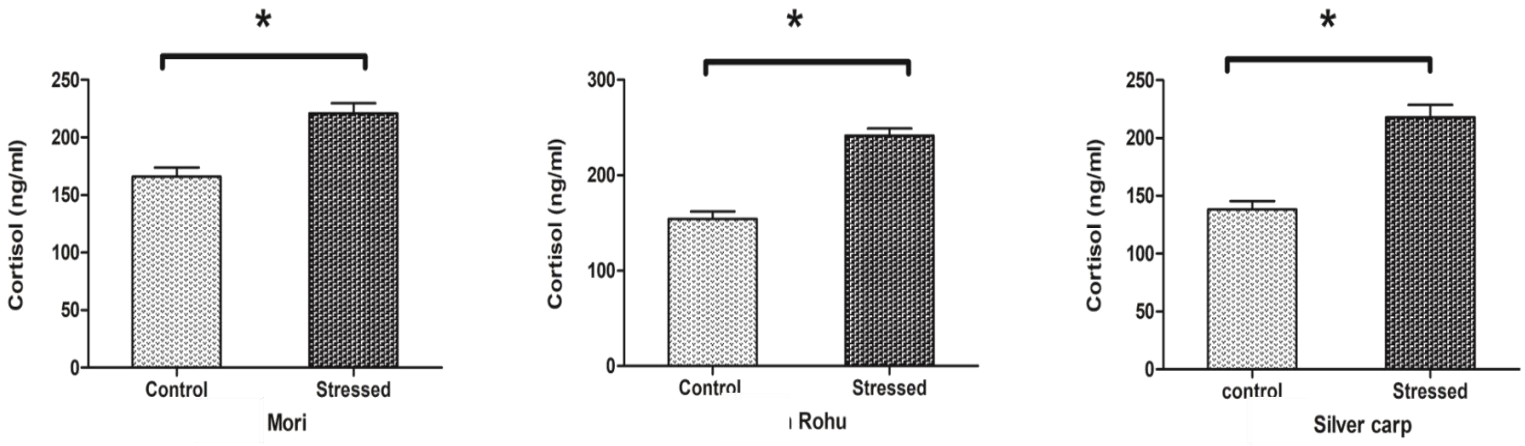


Fig 15: Changes in the post stress concentration (ng/L) serum cortisol in the studied fish species. The bar shows the data as mean ± S.E. (n=3). T. test indicated significant differences among the controlled and stressed fish species. Starts on the bars showing the level of significance. ($P < .001$).

Discussion

It is a well-known fact that stress is real and perceived challenge to fish ability to meet its metabolic needs. Although often the effect of natural stress on metabolism is noticeable, overgrowth is not always apparent (Birnie-Gauvin *et al*., 2017). To study the stress and their effects it is always difficult to identify the chronic stress level limitations, thus, to determine the level of stress sometimes the cortisol level acts as the first indicator (Nakano *et al*., 2020). Describing the apparent discrepancy between high level of cortisol and low level of growth, the question may be raised as to whether cortisol is the most appropriate parameter in this regard for measuring chronic stress (Wojtaszek *et al*., 2002; Burgos-Aceves *et al*., 2016).

Few years ago, the concept of animal stress was suppressed and did not accept the effects of short-term changes in cell structure, hematology and tissue, as a major characteristic syndrome and environmental disorders in humans. Seyle's description of stress as a "subtle (normal) effect of any need on the body" now encapsulates homeostasis in a broad sense, including all levels of succession to a network biological system (Vodougnon *et al*., 2018). The differences in stress responses are therefore different internally and pressures become many in terms of typology, source and results, and the answers each person seeks to deal with the disruption. In fish, during the course of the transition period after stress depends largely on certain factors, including depressive experiences in early life, vertical transfer of depressive phenotypes, individual phenotypic plasticity level, durability and diversity of epigenetic networks related to environmental changes, and internal behavioral responses (personality / personality) of the individual (Backström & Winberg. 2017; Burgos-Aceves *et al*., 2019). Any there may cause

disturbance and change either in the levels of different hormones inside the body of animal or total cell count of blood cells or the concentration of plasma (Niemikoski *et al*., 2020).

The practices used during netting and harvesting of hatchery reared fish frequently exaggerate stress, consequently, reduceing their abilities to respond and protect themselves from different predators (Näslund *et al*., 2013). Thus, during the stress response the animal may seek the shelter of favorable environment for their survival. Results of current study also showed that lower levels of hematological parameters and higher concentration of serum cortisol of the stressed fish as compared to control.

There are different parameters which are being used to determine the sub-lethal effect of different stressor on fish. To determine the level of stress via netting stress. First of all, we measure the hematological indices of controlled and stress fish were determined. The results showed that in stress fish the level of hematological indices is significantly different from control fish. There were different parameters which were studied as hematological indices indicated the highly significant increase /decrease in hemoglobin level, Hematocrits level, MCH level, MCHC level, MCV, platelets, RBCs level and WBCs level and etc. During our investigation, the lower the level of hematocrit and hemoglobin might be because of red blood cells shrinking. The red blood shrinked as venomous action of lindane on erythropoietic tissue (Saravanan *et al*., 2011; Islas-Flores *et al*., 2011; Harabawy & Ibrahim. 2014).

Studied fish species induced significant decrease in hemoglobin level (Fig 2), hematocrits level (Fig 3), MCH level (Fig 4), MCHC level (Fig 5) and MCV (Fig 6), between two groups of all studied species. Previously study by Rifkind *et al*., 1980 and Kori-Siakpere and Ubogu. 2008 reported that the level of MCV MCH, MCHC significantly

decreases due to exposure of some pesticides in fish. It means that due to stress condition the level of abovementioned parameters decrease as compared to non-stressed fish, this study was in accordance with our results. The hemoglobin is performing an important function in the body of an animal as it is used to transport oxygen to peripheral part of fish from the gas exchange parts (Vitagliano *et al*., 2004; De Souza and Bonilla, 2007). MCH value is related to two other values, mean corpuscular volume (MCV) and mean hemoglobin corpuscular concentration (MCHC). Collectively, MCH, MCV, and MCHC are sometimes referred to as red blood indices (Ribeiro *et al*., 2006; Pereira *et al*., 2013).

Mean corpuscular volume (MCV) is measured as normal size of RBCs and MCH results totally depends on the effect of MCV. This phenomenon happened as more hemoglobin is present in larger red blood cells and vice versa (Santhakuma *et al*., 1999; Alwan, 2009). The difference between MCH and MCHC is that the MCHC rate assumes the volume or size of a red cell account while MCHC does not (Jones *et al*., 2005; Saravanan *et al*., 2011). The lower level of MCHC during stress condition might because of decrease in level of synthesis of HB (Nikinmaa, 1982; Nussey *et al*., 1995) Our result agreed with previous reported study of Fletcher 1975; Caldwell and Hinshaw 1994; Houston *et al*., 1996; Witeska. 2005). They also reported that the hemoglobin, MC, MCV as well as MCHC level changed due to stress.

In our study the level of RBCs decreased significantly in stressed fish as compared to control fish. The lower level of RBCs in stressed fish may happen due to inhibition of erythropoiesis. The impaired osmoregulation and damage of gill might be another possible reason of RBCs decline in stressed fish as compared to control (Islas-Flores *et al*., 2009; Saravanan *et al*., 2011). The similar study conducted by Caldwell & Hinshaw. (1994) and

Saravanan *et al*. (2011). They also highlighted the decline of RBCs in stressed fish. There also might be other possibilities like either the inhibition of hemoglobin synthesis and RBC formation or the destruction of erythrocyte (Jenkins *et al*., 2003; Ramesh and Saravanan, 2008).

In the present study, the stressed fish group of all studied species showed the reduction of WBCs level as compared to control. The reduction in the level of WBCs leads to lower the nonspecific immunity of fish as immunological function of fish can be disturbed with the exposure of various stress conditions. Ni *et al*. (2014) and Al-Deghayem *et al*. (2017) also reported the decline of WBCs due to some stress condition in fish species. Our results were inurement with the already described studied (Ni *et al*., 2014; Al-Deghayem *et al*., 2017). Similar results have already been reported by klesius *et al*. (1999); Jenkins *et al*. (2003); Verma, (2007) and adewoyw, (2010).

There are highly significant differences observed in platelets (Fig 8) between control and stressed studied fish species. In stressed fish species the level of platelets decreases as compared to control fish. This indicated that in case of injury the stressed fish can die easily as the function of platelets is to prevent bleeding during injury (Meseguer *et al*., 2002; Chikashige & Iwasaka, 2018; Olas, 2020). Our findings were alike to findings described by other researchers after study the response of fish after stress (Hasan *et al*., 2015). Our results about hematological indices were strongly supported by similar point of view of different scientist about stress on fish species (Yekeen and Fawole 2011; Ramesh and Saravanan 2008; Adhikari *et al*. 2004; Tamizhazhagan and Pugazhendy. 2015).

Complete blood counts and chemical plasma profiles are important diagnostic tools, with laboratory procedures and guidelines used to treat aquatic animals. Like non-

mammalian earth partners, fish erythrocytes are nucleated, and many leukocytes exhibit morphology alike to blood films: thrombocyte, monocyte, basophils, and lymphocytes (Roche & Bogé. 1996 Martínez-Álvarez *et al*., 2005, Chien *et al*., 2003).

Lymphocytes of fish are tiny circular cells with a high N: C ratio and a smooth cytoplasm line around a large horizontal circular nucleus. The lymphocytes of certain fish, especially the elasmobranch species, often appear untreated with "blebs" or skin peeling (Van Weerd & Komen.1998; Kiron *et al*., 2004; Vinagre *et al*., 2012). Some authors continue to classify them as large size, although performance differences are not yet understood (Vinagre *et al*., 2012).

The morphology of monocytes of fish is somehow similar to the lymphocytes of the fish. The structure of monocytes is little bit different as its nucleus is oval, round, or lobed with compact chromatin which is usually loosely attached with it. It also contains blue cytoplasm and vacuole. A large circular cell that is as white as light blue to gray and is filled with small rod-shaped granules. The nucleus can be circular or integrated several times (Magnadotti. 2010, Di Marco *et al*., 2008; Segner *et al*., 2012). The lymphocytes level increased in stressed fish as compared to control in our study. The increased level of lymphocyte occurs because during stress reduces white blood cells count particularly lymphocytes (Witeska, 2005).

There was no significant differences in Heterophils (Table 1) Lymphocytes (Table 2), Eosinophils (Table 4) in Rohu and Silver carp fish species while a significant difference was found between stressed and controlled Mori fish. While the Monocytes cell count was not significantly different in Mori but in Rohu and silver carp the level was significantly different between controlled and stressed (Table 3). The Basophiles cell count was

significantly difference in Mori and silver carp but found non-significant in Mori Fish species (Table 5). Basophils and eosinophils are rarely reported in teleost boundary blood (Tavares-Dias. 2006); however, this study revealed cells in a time of structural stress or morphology of altered blood cells. The morphological characteristics of basophils observed in all stressed species differ in control. Our study was consistent with research by Tavares-Dias and Moraes. 2007). The presence of eosinophils in the studied fish smears is reported to be equally rare (Campbell and Ellis. 2007). In conclusion, leukocyte morphology also changed in stress fish compared to control in all cases.

In our investigation, the swelling in erythrocytes is observed which agreed to previously reported study of Witeska, 2005. He reported that due to osmotic disturbance in fish during stress the swelling in erythrocytes occurred.

To study the effect of netting stress on fish cortisol no specific study was conducted before, although there are lot of studies published to check the effect different stress response on cortisol level of fish. In our investigation, the cortisol level was significantly higher in stressed fish as compared to control. The cortisol also known as stress hormone which increases during an emergency (Goikoetxea *et al*., 2017; Sadoul & Geffroy. 2019: Miller *et al*., 2019). The change in the concentration of cortisol cause to increase the concentration off plasmatic glucose segregation. So as the concentration of cortisol was higher in stressed as compared to control then indirectly the concentration of glucose was also higher in stressed (Aliko *et al*., 2018; Uçar *et al*., 2020; Fazelan, *et al*., 2020). Results from present investigation are in accordance with the study of Aliko *et al*., 2018. Their results showed that the level of cortisol increased in fish exposed to stress of manganese. Similar results were reorted by Karaytung *et al.* (2010). After their experiment on stress by

different concentrations of Cadmium. During the stress of fish, the cortisol released from anterior pituitary gland Wojtaszek *et al*. (2002) and Burgos-Aceves *et al*. (2016) described that through the process of gluconeogenesis and glycogenolysis the cortisol activates which causes the increase of catecholamines release with the help of chromaffin cells.

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