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A comparative study of the hematological parameters of *Clarias batrachus* (Linnaeus, 1758) and *Clarias gariepinus* (Burchell, 1822) from North Bihar, India

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Abstract
The genus *Clarias* (Family: *Clariidae*; order: *Siluriformes*) incorporates air breathing Clariid catfishes, having ability to survive out of water for longer duration. *Clarias batrachus* (Linnaeus, 1758) [24], commonly called as 'Magur', is an endogenous species of India and is very popular in Asiatic countries because of its taste and medicinal properties. *Clarias gariepinus* (Burchell, 1822), an African catfish commonly called as 'Thai magur, is deadly carnivorous exotic species introduced to India through various trade means. This exotic species has undergone tremendous adaptation to the physicochemical condition of the aquatic bodies of Bihar, India, resulting in a considerable decline of the native species *C. batrachus* from the local fish market. The aim of the present study was to investigate and establish reference ranges by comparing haematological indices of two closely allied fresh water air breathing species of family *Clariidae i.e. C. batrachus* and *C. gariepinus* from the wetland of North Bihar. In the present study hematological indices like red blood corpuscles (RBC), total and differential white blood corpuscles (WBC), hematocrit (Ht), haemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) were measured in the blood samples of both the fish species using standard methods. The values were determined as mean ± SEM for thirty (n=30) replicates in each parameter. One-way analysis of variance (ANOVA) was used to compare the haematological findings between and within these two species of catfishes. Most of the haematological parameters differ non-significantly ($p < 0.05$) between *C. batrachus* and *C. gariepinus* except haemoglobin and leucocytes, which showed highly significant differences. The findings of the present study will give an insight of the actual reference range of various haematological parameters and interspecific variations between these two species with regard to the blood profile. Furthermore, these indices will provide reliable information on the metabolic disorders, deficiencies and chronic stress status of the fishes before they become apparent in a clinical setting. It will definitely help in raising the status of aquaculture in state of Bihar.

Keywords: Blood, *Clarias batrachus, Clarias gariepinus,* Haematological analysis, North Bihar, India

Introduction
Health and physiological status of the fish can be assessed effectively by considering haematological indices as bio-indicators (Clauss *et al.*, 2008; Adeyemo *et al.,* 2009; Siwicki *et al.,* 1994) [14, 2, 52]. In addition, these parameters play a significant role in the diagnosis of disease and assessment of metabolism of fishes living in different ecological environments by reflecting the correct changes within them (Clarence and Hickey, 1982; Cengizler and Şahan, 2000) [13, 12]. The exhaustive studies made on the haematological indices have successfully been used to establish the phylogenetic relationship among certain species of fishes (Cameron 1970; Larsson *et al.* 1976; Atkinson and Judd 1978; Putnam and Freel, 1978; Filho *et al.* 1992) [10, 33, 5, 45, 92]. The blood in fishes measures 1.3-7% of their total body weight. It ensures gas exchange between the organism and the environment. It plays a detrimental role in accomplishment of different metabolic processes. It facilitates the transportation of all kinds of nutrients, enzymes, hormones, neurotransmitters and all metabolic intermediaries in the different parts of body, apart from helping in transporting nitrogenous wastes outside the body of the fish. The functional status of the oxygen carrying capacity of the blood stream in fish can be effectively assessed by several hematological indices such as haematocrit (Hct), hemoglobin (HB), red blood cells (RBCs) *etc.* Hence these indices are convincingly used as bio-indicators of the patho-physiological condition or sub-lethal stress response in fish to

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various endogenous or exogenous changes in the aquatic environment (Cataldi *et al.* 1998; Belanger *et al.* 2001; Shah and Altindağ, 2004a; Adhikari *et al.,* 2004; Radu *et al.,* 2009; Smith, 1945) [11, 7, 49, 3, 46, 53].

Fishes being poikilothermic vertebrates, experiences considerable changes in the metabolic contents of the blood in response to change in various environmental factors *viz.* bacteria, parasites, water temperature, oxygen content, pH *etc*. (Bullis, 1993) [9]. Besides, the blood components exhibit a wide variety of genetic and physiological variations. The aquatic biotope, fish species, age, sexual maturity and health status influence the changes in haematological parameters (Blaxhall, 1972; Patriche *et al.* 2011; Radu *et al.* 2009) [42, 46]. An ideal reference range of various blood components is considered as reliable descriptors of healthy fish under natural conditions (Cataldi *et al.,.* 1998) [11]. Any kind of evaluation of haematalogical parameters is based on the normal reference range under ideal natural condition.

Clarias batrachus, commonly known as 'magur' is an endogenous Asian species, which is commercial highly significant in India and other Asian countries including Bangladesh, Thailand, Vietnam, Malaysia and Indonesia due to its excellent nutritional status with high Vitamin D content, low level of omega-3 fatty acids and a much higher proportion of omega-6 fatty acids as well as its high medicinal and therapeutic value (Mollah & Karim, 1990; Hossain *et al.,* 2006; Islam *et al.*, 2007) [39, 24, 27]. After being originated in India, it spreads globally (Kottelat, 2001; Lever, 1996) [32, 34]. Four sub species of *Clarias batrachus* are known to be prevalent in South East Asia, Java and India as *C. batrachus; C. aff. batrachus "Indochina"*; *C. aff. batrachus "Sundaland"* and *Clarias magur (Hamilton,* 1822) (Ng & Kottelat, 2008) [40]. The invasion of a morphologically alike and deadly carnivores African species *Clarias gariepinus* (Burchell, 1822) in India and their ability to hybridize naturally with the native species and excessive predation on them have potentially threaten the survival of *Clarias batrachus* (Thakur, 1998; Khedkar *et al.,*2010) [60, 28]. The culture and import of *C. gariepinus* in India was totally banned in 1997 by the Ministry of agriculture in India (Gopi & Radhakrishnan, 2002) [20]. But due to prolific breeding ability, excessive acclimatization in the Indian condition and simplicity in rearing, this exotic species has compelled the fishermen in Bihar and other states of India to culture it on large scale, setting aside the ban and restriction imposed by the ministry of agriculture, Government of India (Singh & Lakra,2006).

The purpose of the present study was to characterize base-line concrete haematological indices of *Clarias batrachus* and *C.gariepinus*, and to establish a reference point that can be efficiently applied for monitoring of fish health, disease status, reproductive potentials and various eco-toxicological studies. The finding of the present study can be correlated with the advanced mitochondrial genome analysis of these two closely allied species of family *Clariidae*. It was assumed that a systemic study of their haematological indices may provide information about interspecies differences between them and adaptations of the exotic species to the aquatic conditions of Indian subcontinent.

Materials and methods
Biological sampling and acclimatization
A total of 60 adult specimens, 30 each of *C.batrachus* and *C. gariepinus* ranging from 60-110 gm ranging from 60-110 GM and size between 4.6"-7.1" were collected from Jalalgarh fish farm, Purnia, Bihar, India during June 2019. Fishes were at first brought to laboratory and were disinfected with 0.1% $KMnO_4$ solution and kept in different sized large plexi glass aquaria (capacity 50L, 80 L and 100L) having DE chlorinated, aerated tap water at normal temperature and pressure. Fishes were acclimatized to the ideal laboratory condition for 15 days as per standard method of APHA (2005). Fishes were fed *ad libitum* and the fresh water was changed regularly in the morning hours of every day. To maintain normal temperature and pressure of water, cooler and exhaust were used around the aquarium.

Collection of blood sample
Fishes were cold anaesthetized using MS222 (Imanpoor *et al.*, 2010) [26]. The peripheral blood was collected by puncturing the posterior caudal vein with heparin treated 21 gauge x 0.5 inch needle fitted with 2 ml syringe as per the standard protocol of Schmitt *et al.* (1999).The collected blood samples were then transferred into microtubes, containing EDTA (ratio 1.26 mg/0.6 ml) as the anticoagulant agent and were gently mixed by carefully turning it upside down. The collection tubes were then kept in the sampling boxes containing ice. The time elapsing from capture to blood withdrawal was less than 5 min.

Haematological analysis
In the experimental protocol, estimation of Haemoglobin (HB) was done with a hemoglobin test kit (Diagnova, Ranbaxy, India) using the cyanmethemoglobin method (Blaxhall and Daisley, 1973; Tanyer, 1985) [8, 58]. "Microhematocrit Technique" was employed for determination of Hct (Blaxhall and Daisley, 1973; Konuk, 1981; Şahan and Cengizler, 2002) [8, 31, 47]. The total red blood cells (tRBCs) were counted using an improved Neubaur haemocytometer (Shah and Altindağ 2004a) [49]. Blood was diluted 1:200 with Hayem's fluid (Mishra *et al.*, 1977) [38]. Erythrocytes were counted in the loaded haemocytometer chamber and total numbers were reported as 106 mm^3 (Wintrobe, 1967) [63]. The erythrocyte indices were calculated according to the following formula: Mean Corpuscular Volume (MCV) (µ3/femtolitre) = HCT (%) ÷ RBC (106/ mm3) x 10; Mean Hemoglobin Concentration (MCH) (PG/picogram) = HB (g/100 mL) ÷ RBC (106/ mm3) x 10; Mean Corpuscular Hemoglobin Concentration (MCHC) (%) = HB (g/ 100mL) ÷ HCT (%) x 10 (Kocabatmaz and Ekingen, 1984) [29].

Total count of WBC Total white blood cells (WBC) were counted using an improved Neubaur haemocytometer (Shah and Altindağ 2005; Mgbenka *et al.*, 2003) [50, 37]. Blood was diluted 1:20 with Turk's diluting fluid and placed in haemocytometer. 4 large (1 SQ mm) corner squares of the haemocytometer were counted under the microscope (Olympus) at 640 X. The total number of WBC was calculated in mm3 x10^3 (Wintrobe, 1967) [63].

Statistical analysis
Results are presented as mean with standard error of mean (SEM) of thirty replicates in each case. The results were subjected to student's t-test. One Way analysis of Variance (ANOVA) was used to determine significant differences in between experimental groups and significance levels of these differences of all parameters measured (Hayran and Özdemir, 1995) [23]. The level of significance was considered at $p < 0.05$. SPSS 10.0 software was used for statistical analyses.

Results

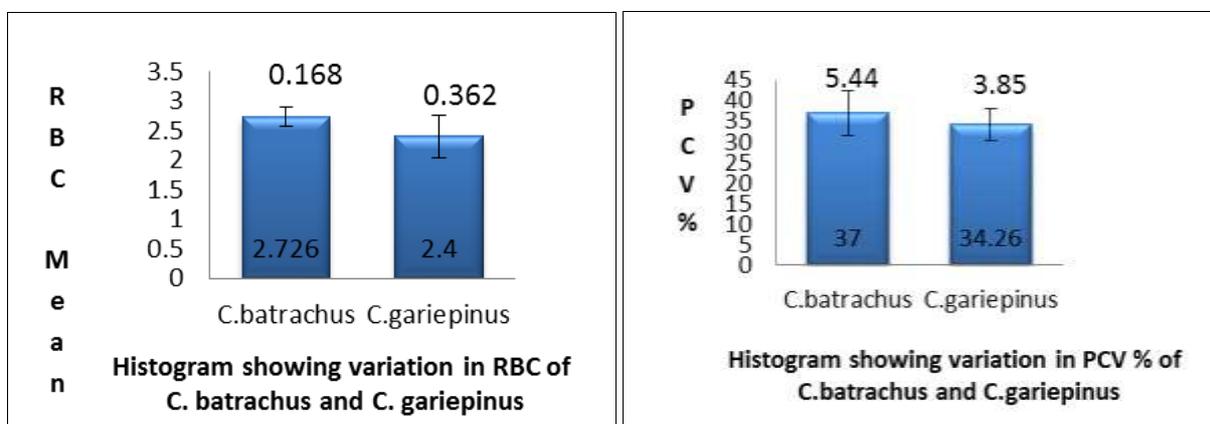The haematological value obtained for the two species *i.e.* *Clarias batrachus* (Linnaeus, 1758) [24] and *Clarias gariepinus* (Burchell, 1822) have been presented as Mean ± SEM in each case in Table – I.

Table 1: Hematological parameters of *Clarias batrachus (*Linnaeus*, 1758)* [24] and *Clarias gariepinus* (Burchell, 1822)
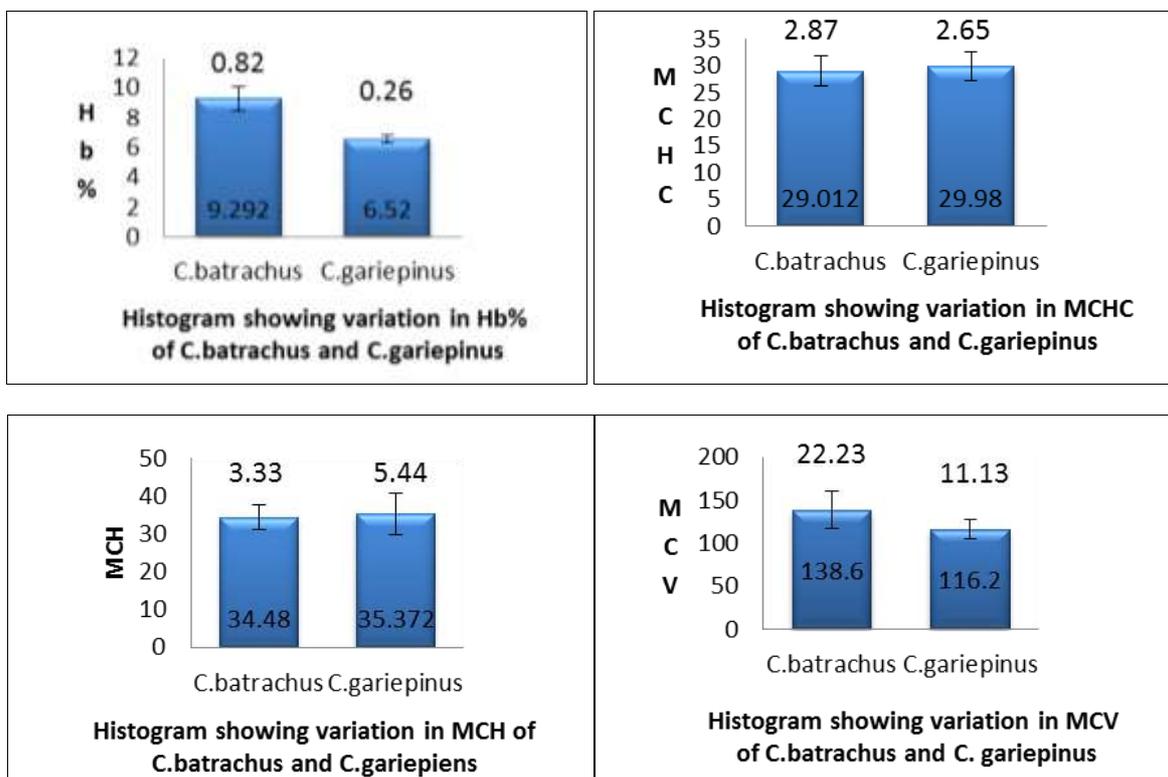
Demonstrated parameter	Sample size	Clarias batrachus		Clarias gariepinus		Calculated F value	Table F value
		Mean	SEM	Mean	SEM		
RBC (x10^6/ µl)	30	2.726	0.168	2.40	0.362	0.803	5.31
PCV (%)	30	37	5.44	34.26	3.85	0.168	5.31
Hb (g/dl)	30	9.29	0.82	6.52	0.26	10.25*	5.31
MCV(fl)	30	138.6	22.23	116.2	11.3	0.811	5.31
MCH(PG)	30	34.48	3.33	35.37	5.44	0.019	5.31
MCHC (g/dl)	30	29.01	2.87	29.98	2.65	0.061	5.31
WBC (x10^3/ µl)	30	106.56	23.41	70.82	16.68	1.545	5.31
Lymphocyte (%)	30	64.56	4.27	49.22	6.46	3.917	5.31
Monocyte (%)	30	1.6	0.40	3.0	0.31	7.53*	5.31
Eosinophils (%)	30	0.60	0.39	0.80	0.67	0.13	5.31

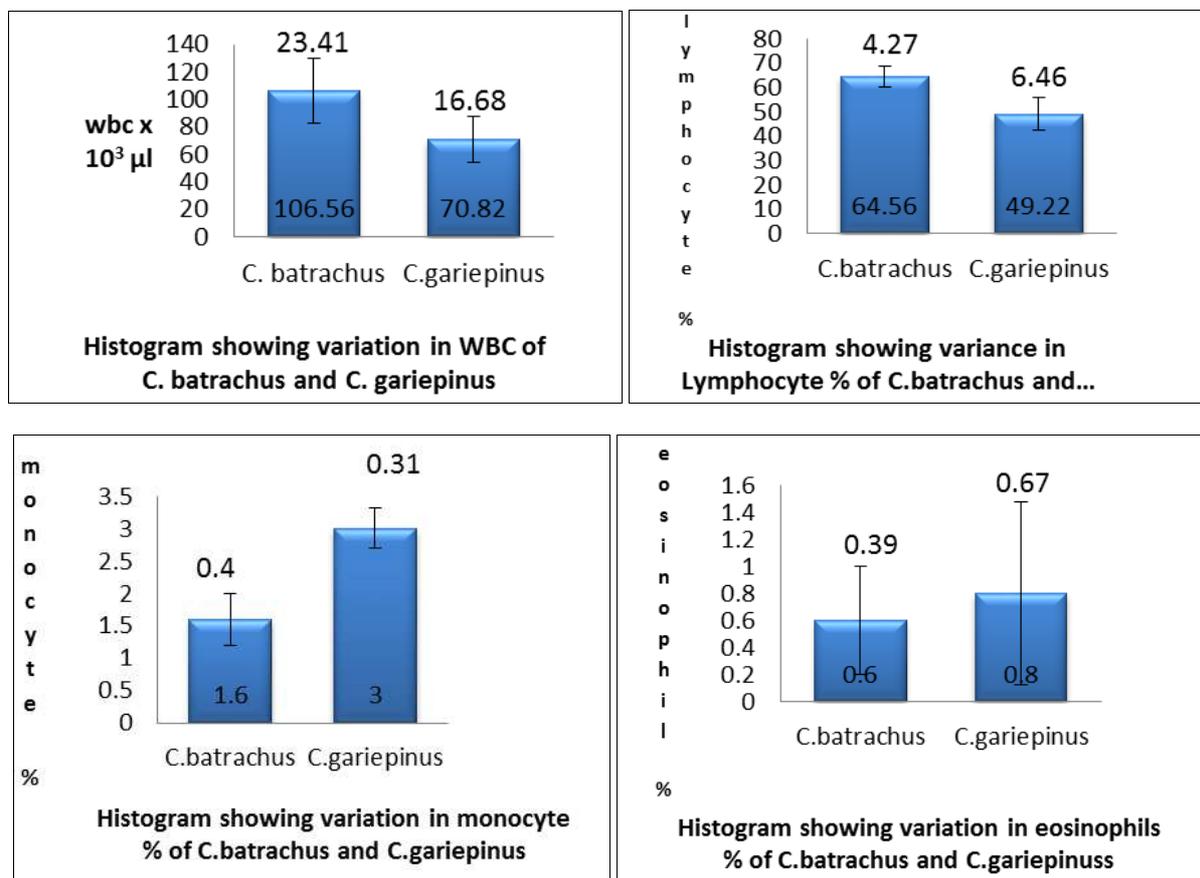Figures in each parameters have been shown as Mean ± SEM, the sample size in each in case is n = 30, * significant at $p <$ 0.05. One way analysis of Variance was done. Calculated value has been compared with table value.

Histogram showing variation in RBC of C. batrachus and C. gariepinus

Histogram showing variation in PCV % of C.batrachus and C.gariepinus

Graph 1

Histogram showing variation in Hb% of C.batrachus and C.gariepinus

Histogram showing variation in MCHC of C.batrachus and C.gariepinus

Histogram showing variation in MCH of C.batrachus and C.gariepiens

Histogram showing variation in MCV of C.batrachus and C. gariepinus

Graph 2

Graph 3

Discussions

The blood is considered as a mirror in which, majority of the vital processes of an organism taking place, are reflected. The blood of teleost efficiently reflects the essence of the metabolic processes in fishes (Hawkins, 1971) [22]. Besides, toxicological research and environmental monitoring studies have consistently included fish blood as a possible indicator of patho-physiological changes in fishes. Therefore, it is incorporated as essential component of the fisheries management in India (Gupta and Gupta, 1981; Sancho *et al.*, 2000; Barcellos *et al.,* 2003) [21, 48, 6]. The present study was aimed to establish a correlation among two morphologically identical closely allied species of catfishes *i.e. Clarias batrachus (*Linnaeus, 1758*)* [24] and *C. gariepinus* (Burchell, 1822) of family-*Clariidae* and order-*Siluriformes*, if any, with special reference to haematological parameters. The present study also deals about variations among blood parameters in these two closely related species. Functional status of the oxygen carrying capacity of the bloodstream in fishes can be assessed by a number of hematological indices such as haematocrit (HCT), hemoglobin (HB), Red blood cells (RBCs), MCH, MCHC, MCV, *etc.*

RBCs are the most numerous (98-99%) blood cells in fish (Fange 1994). In the present study, the level of RBCs in control *C.batrachus* and *C.gariepinus* were recorded as $2.72 \pm 0.168 \times 10^6$/ µl and $2.40 \pm 0.36 \times 10^6$/ µl respectively with an average range of $2.40\text{-}2.80 \times 10^6$/ µl. The calculated F value is 0.803, which is much lower than the table value. Hence the difference in the RBC count in both the species are not significant at $p < 0.05$. However a slight difference in RBC count in both the species may be correlated to the difference in the size and swimming activities (Soldatov, 2005) [54]. Maheshwaran *et al.*, (2008) [36] has determined the total

erythrocytes count as $177\pm0.014\times10^6$/ µl. Adedeji and adegbile (2011) [1] have reported total RBC count in *Clarias gariepinus* as $1.77\pm0.014\times10^6$/µl.

Fish erythrocytes are ellipsoidal and contain nucleus. However, erythrocyte count in fish is strongly dependent on environmental conditions, mostly on temperature and dissolved oxygen level. Therefore, the seasonal changes in erythrocyte numbers pose difficulties in establishing the hematologic reference values (Luskova 1997) [35]. In aquatic environment, O_2 availability may considerably vary, and fish react to hypoxic conditions with elevation of blood oxygen carrying capacity – a rapid release of stored cells from a splenic reservoir or an increase in erythropoietic rate (Houston, Roberts, and Kennington 1996) [25]. The metabolic rate in poikilotherm animals is greatly affected by ambient temperature which causes seasonal alterations in fish red blood cell parameters: fishes living in higher temperatures showed higher RBC (Sopińska 1983; Svetina *et al.* 2002) [55, 57]. Besides, water, pH, salinity affect erythrocyte count in fish.

RBC indices basically include MCV, MCH and MCHC, haemoglobin (HGB) and haematocrit (HCT) value. Mean cell (or corpuscular) volume (MCV) refers to the average size of the RBCs constituting the sample. Mean cell hemoglobin (MCH) represents to the average weight of hemoglobin in the RBCs in the sample. Mean cell hemoglobin concentration (MCHC) refers to the average concentration of hemoglobin in the RBCs contained within the sample. The saturation of an organism with oxygen is reflected by the concentration of haemoglobin and the organism itself tries to maintain them at homeostasis. A decrease in the erythrocyte count and in the percent of haematocrit indicates the worsening of an organism state and developing anaemia. The calculation of MCV, MCH

and MCHC were directly linked with reference to RBC, HCT and Hgb.

Erythrocytes of fish are sensitive to various environmental impacts but, according to Vosylienė (1999) [61], the basic quantitative red blood parameters (e.g. hematocrit, erythrocyte count or hemoglobin concentration) in fish tend to remain stable due to considerable compensatory potential. The damage of erythrocytes caused by adverse environmental factors may be compensated by the release of new cells from the spleen (erythrocyte storage organ) or/and head kidney (hematopoietic organ), which is indicated by increase in the frequency of immature cells in peripheral blood. In the present study, the level of MCV in control *C. batrachus* and *C. gariepinus* were recorded as 138.6 ± 22.23 FL and 116.2 ± 11.3 FL respectively with a range of 110-160 fl. The calculated F value is 0.811, which is much lower than the table value *i.e.* 5.31. It is non- significant ($p < 0.05$). However, the mean MCV for *Clarias gariepinus* from Nigeria have been reported as 99.575 ±3.64 FL, ranging between 88-160.90 FL (Adedeji and Adegbile, 2011) [1].

The level of MCH in control *C. batrachus* and *C. gariepinus* were recorded as 34.48 ± 3.33% and 35.37 ± 5.44% respectively, with an average range of 30-42%. The calculated F value is 0.019, which is much lower than the table value. It is non- significant ($p < 0.05$). Although the MCH in *C. gariepinus* were earlier reported to be in range between 25.10-48.90pg with a mean value of 31.345±1.20pg (Adedeji and Adegbile, 2011) [1].

The level of MCHC in control *C. batrachus* and *C. gariepinus* been recorded as 29.01 ± 2.87% and 29.98 ± 2.65% respectively, with an average range of 26-32%. The calculated F value is 0.061, which is much lower than the table value. The difference in the concentration of MCHC in both the species are non- significant ($p < 0.05$). The MCHC in *C. gariepinus* was earlier reported to vary in between 26-32, with a mean value of 30.7±0.41 (Adedeji and Adegbile, 2011) [1].

The packed cell volume (PCV) is a measurement of the proportion of blood that is made up of cells. The value is expressed as a percentage or fraction of cells in blood. It is one of the indicators of fish health and sometimes it is also called as hematocrit. The level of PCV % in control *C. batrachus* and *C. gariepinus* were recorded as 37 ± 5.44 and 34.26 ± 3.85 respectively, with an average range of 30-44%. The calculated F value is 0.168, which is much lower than the table value. It is non- significant ($p < 0.05$). Previously recorded mean PCV for *Clarias gariepinus* was 30± 1.09%, with a range of 18-37% (Adedeji and Adejbile, 2011) [1].

The level of haemoglobin (g/dl) in control *C. batrachus* and *C. gariepinus* were recorded as 9.29 ± 0.82 and 6.52 ± 0.26 respectively. The calculated F value is 10.25, which is much higher than the table value. It is highly significant at $p < 0.05$. The higher concentration of haemoglobin in *C. batrachus* is linked with the high metabolic activities of the fish (Eisler, 1965) [65]. An abnormally high concentration of haemoglobin in *C. batrachus* (75.00±4.04 g/dl) have been earlier reported (Maheshwaran, 2008) [36]. The mean haemoglobin concentration in *C.gariepinus* have been earlier reported as 9.28±0.42 g/dl (Adedeji and Adejbile, 2011) [1].

White blood cells play a major role in the defense mechanism of the fish and consist of granulocytes, monocytes, lymphocytes and thrombocytes. Granulocytes and monocytes function as phagocytes to salvage debris from injured tissue and lymphocytes produce antibodies (Ellis *et al.*, 1978; Wedemeyer and Mcleay, 1981) [62]. The number of leucocytes determines the extent of immune responses and the ability of the animal to fight infection (Douglass and Jane, 2010) [15]. Higher levels of WBC in any species are the indication of their efficient way of fighting with pathogen than other species. Fish thrombocytes are blood phagocytes that form a protective barrier (Tavares-Dias and Moraes, 2004; Prasad and Charles 2010; Prasad and Priyanka 2011) [59, 44, 43]. They express a large number of surface and intracellular molecules which are involved in immune function (Kollner 2004) [30]. They represent a link between innate and adaptive immunity (Passantino *et al.* 2005) [41] and participate in defense mechanisms efficiently (Stosik *et al.* 2001) [56]. In the present study, the level of WBCs in control *C. batrachus* and *C. gariepinus* were recorded as 106.56 ± 23.41x10³/ µl and 70.82 ± 16.68 10³/µl respectively, with an average range of 60-115 x10³/ µl. The calculated F value is 1.54, which is much lower than the table value. It is non- significant ($p < 0.05$).

Lymphocytes are most common and variable leucocytes in healthy teleost. An increase in lymphocyte number may be the compensatory response of lymphoid tissues to the destruction of circulating lymphocytes (Shah and Altindağ, 2005) [50]. The level of lymphocyte in control *C. batrachus* and *C. gariepinus* were recorded as 64.56 ± 4.27% and 49.22 ± 6.46% respectively. The calculated F value is 3.91, which is much lower than the table value. It is non-significant at $p < 0.05$. The higher percentage of circulating lymphocytes in *C. batrachus* can be linked to its efficient humoral and cell mediated adaptive immune responses (Douglas and Jane, 2010) [15].

Monocytes in teleosts are the largest cell among different leucocytes, irregular in shape, having eccentric, large, heterochromatic nucleus and abundant slightly basophilic cytoplasm with vacuoles and other membrane bound organelles. In the present study, the concentration of monocyte (%) in control *C. batrachus* and *C. gariepinus* were recorded as 1.6 ± 0.40 and 3.0 ± 0.31 respectively. The calculated F value is 7.53, which is higher than the table value i.e. 5.31. It is significant at $p < 0.05$. True eosinophils are less common in teleost. The level of eosinophils (%) in control C. batrachus and C. gariepinus were recorded as 0.60 ± 0.39 and 0.80 ± 0.67 respectively. The calculated F value is 0.13, which is much lower than the table value. It is non-significant at $p < 0.05$. A concentration of comparatively higher eosinophils might be associated with parasitic eradication, allergy and chronic inflammation.

The present study summarizes some of the salient features of hematologic analysis in two closely allied species of air breathing fish *Clarias batrachus* (Linnaeus, 1758) [24] and *Clarias gariepinus* (Burchell, 1822) of family Clariidae, inhabiting the same aquatic bodies. The reference ranges obtained can be used later, as a sensitive index to monitor various and patho-physiological changes in fish due to xenobiotic stress. It will also help in monitoring both the health status of the fish and the pollution status of the aquatic bodies. Further studies are needed to trace the molecular and genetic basis of the significant variations in the haematological indices of these two closely allied fresh water air breathing fish species.

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