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Original article

Hematological and serum biochemical reference intervals of rainbow trout, *Oncorhynchus mykiss* cultured in Himalayan aquaculture: Morphology, morphometrics and quantification of peripheral blood cells

Naveed Nabi [a], Imtiaz Ahmed [a,*], Gohar Bilal Wani [b]

[a] Fish Nutrition Research Laboratory, Department of Zoology, University of Kashmir, Hazratbal, Srinagar, Jammu and Kashmir 190 006, India
[b] Faculty of Fisheries, Sher-e-Kashmir University of Agricultural Sciences and Technology, Rangil, Ganderbal, Jammu and Kashmir 191201, India

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ABSTRACT

De novo reference intervals (RIs) for a total of thirty two hematological and serum biochemical attributes were established for rainbow trout (*Oncorhynchus mykiss*) cultured in Himalayan aquaculture system. For this purpose, long term assessment of hemato-biochemical parameters was carried over a period of one year from March 2019 to February 2020 and a total of 444 blood samples were analysed. Blood examination results were recorded systematically and reference intervals were established, notably for erythrocyte parameters: hematocrit (Hct) 29–40%, hemoglobin 8.32–12.28 g/dL, red blood cell (RBC) count 1.01–2.04 ($\times 10^6$/mm3); leukocyte parameters (x 10^3/mm3): total leukocytes 31.32–90.60, neutrophils 4.21–18.85, total lymphocytes 20.55–63.63, small lymphocytes 14.86–46.50, large lymphocytes 6.35–22.34 and monocytes 1.22–7.56; thrombocyte count 23.00–68.00 ($\times 10^6$/mm3). RIs were also established for red blood cell indices, vital serum constituents involved in carbohydrate, protein, lipid and nitrogen metabolism including the less known, diagnostically important, serum enzymes and electrolyte concentrations. Principal component analysis revealed that certain serum components were more efficient at distinguishing between the life stages (juvenile, adult) of fish by explaining about 92.7% of variation in the whole dataset compared to the principal hematological components which explained only about 80% of the variation. Significant ($P < 0.05$) differences were noted for RBC count, total leukocyte count (TLC), total protein, total cholesterol and uric acid with respect to the sex of fish. Moreover, clearly differentiable morphometric and morphological attributes were also noticed among erythrocytes, leukocytes (lymphocytes, neutrophils and monocytes) and thrombocytes. To our knowledge, the present study is the first of its kind that elucidates blood chemistry of cultured rainbow trout, *O. mykiss* in accordance to the guidelines framed by the American society of veterinary clinical pathologists (ASVCP). RIs reported here can help monitor the fish health status by improving the use of non-lethal diagnostics in piscine medicine.

1. Introduction

In the recent years, aquaculture in India has become an area of increasing interest attracting both public and private entrepreneurship (Singh et al., 2016). The country ranks second in aquaculture production after China in terms of the value of its aquaculture produce. In Asia-Pacific region, rainbow trout is an important cultured fish species and contributes significantly to the freshwater aquaculture production (FAO, 2018). Rainbow trout, *Oncorhynchus mykiss*, an exotic fish to the Indian Himalayas traces back its introduction into this region in 1912 by Mr. F.J. Mitchell (Mitchell, 1918). The tremendous adaptability of this fish to the regional environment has been a key factor for its uniformly flourished culture across Himalayan India. Despite, the current production being far low than the actual potential, a predominant contribution from this region has led to a decadal increase of 31% with around 602 tons of annual trout production across the country (Sehgal, 1999: Singh et al., 2017; FAO, 2018).

* Corresponding author.
E-mail address: imtiazamu1@yahoo.com (I. Ahmed).

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Hemato-biochemistry is a promising area in fish physiological research, primarily due to its substantial role in clinical pathology. Hemato-biochemical investigations provide valuable diagnostics to predict the well-being status of cultured fish species (Fazio, 2019) especially, the blood indices are considered to be the vital physiological markers for examining the stress responses in fish (Seibel et al., 2021) due to disease (Rehulka, 2003; Clauss et al., 2008), toxicants (Hrubec et al., 1997; Groff and Zinkl, 1999), parasitic infestation (Davies and Johnston, 2000; Woo, 2003), nutrient deficiency (Groff and Zinkl, 1999Khan and Khan, 2021), environmental stressors (Cnaani et al., 2004; Parrino et al., 2018), inappropriate stocking density (Refaey et al., 2018), farm practices (Uiuiu et al., 2021) transportation and handling (Refaey and Li, 2018; Ghirmai et al., 2020). Besides acting as stress biomarkers, hematological and serum biochemical parameters of fish are intricately influenced by innumerous factors such as life stage (Fazio, 2019), size (Tran-Duy et al., 2008; Fazio et al., 2020a), sex (Karimi et al., 2013; Parrino et al., 2018), sexual maturity (Santos et al., 2009, Vazquez and Guerrero, 2007), breeding status (Ahmed et al., 2020) species (Parrino et al., 2018) and season (Callozos et al., 1998; Guijarro et al., 2003; Fazio et al., 2020b). Dietary factors such as feeding regime (Ahmed, 2018), quantity and quality of dietary protein (Ahmed and Ahmad, 2020) food availability in natural habitat (Fazio et al., 2013) profoundly influence the energy metabolism that results into considerable fluctuations in the various serum biochemical parameters of fish. In comparison to wild, fish raised under intensive rearing conditions are exposed to a variety of stressors that can affect their growth and welfare (Magnoni et al., 2019). Knowledge regarding the hemato-biochemical changes of fish, reared under intensive conditions, can improve productivity performances, fish welfare and the quality of aquaculture practices (Fazio, 2019; Uiuiu et al., 2021). Therefore, this work signifies a case study about the cultured form of fish which varies from its wild conspecifics.

Reference intervals (RIs) for hematological and serum biochemical parameters have been extensively deliberated in human medicine due to their critical relevance in diagnosing various pathophysiological conditions (Fazio, 2019; Chen et al., 2019). Moreover, this field has been largely extended to veterinary medicine in comparison to fish where it stands largely understudied (Reshma et al., 2020). Many authors have emphasized the difficulties in attributing normal estimates pertaining to the hematology and serum biochemistry in rainbow trout, *Oncorhynchus mykiss* (Wedemeyer and Chatterton, 1970; McCarthy et al., 1973; Wedemeyer and Nelson, 1975; Hille, 1982; Miller et al., 1983; Manera and Britti, 2006; Fazio, 2019). Broad aspects that seem to reason this, are the lack of data quorum, typical physiology of fish which is far seriously influenced by innumerous pre and post-analytical factors, and more importantly the incompliance to the recommended statistical and clinical laboratory guidelines. In addition to the enormously sensitive blood chemistry in fish compared to mammals, the inevitable differences in methodology, size, sex, strain, season, environment, physiological condition, and culture settings largely limit to find out an exhaustive set of blood chemistry reference intervals for rainbow trout in a single study.

Compliance with the statistical recommendations for setting RIs in exotic animal species is a key step after laboratory investigations. Simple usage of the measures of central tendency and standard deviations for defining the RIs is a timeworn and inappropriate method (Hrubec et al., 2000; Knowles et al., 2006). In the past, contrasting approaches have been employed for ascertaining the hemato-biochemical RIs of rainbow trout. Some authors prefer the use of non-parametric statistics (McCarthy et al., 1973; Manera and Britti, 2006) in contrast to those favouring the use of Gaussian methods (Wedemeyer and Chatterton, 1970; Rehulka et al., 2004). Other normal range estimates have achieved comparable results by making use of both the methods (Wedemeyer and Nelson, 1975; Miller et al., 1983).

Although, a lot of work on hemato-biochemical aspects of rainbow trout has been carried out in the past (Wedemeyer and Chatterton, 1970; McCarthy et al., 1973; Wedemeyer and Nelson, 1975 Miller et al., 1983; Rehulka et al., 2004; Manera and Britti, 2006) however, no detailed study that could establish RIs based on recommended clinical and statistical guidelines has so far been carried out. Therefore the purpose of the current study was to define the currently lacking RIs for hematological and serum biochemical parameters of Himalayan cultured rainbow trout using ASVCP protocol (Friedrichs et al., 2012). The possible variations existing within these parameters between different life stages and sex of fish were also analysed. Furthermore, the study also aimed at the identification, quantification and morphometric characterization of peripheral blood cells by using a standard staining protocol followed by the microscopic observation. The study is the first of its kind to provide a complete hemato-biochemical profile of cultured *O. mykiss* that can be used as a reliable tool by fish pathologists for diagnosis and monitoring of pathophysiological abnormalities, particularly, the stress-induced biochemical alterations in this cultured fish species.

2. Materials and methods

2.1. Study site

The Laribal trout farm spread over an area of 2 ha situated in midst of Dachigam National Park (34°09′01.8″N 74°55′10.6″E) Srinagar, India was chosen as a study site (Fig. 1). The farm is one of the Asia's largest trout production centres, equipped with highly sophisticated fish rearing infrastructure. Fish were stocked in well-covered, concrete raceways (dimensions = 30 m × 2.5 m × 1.5 m) fitted with mechanical filters, and supplied with continuous water, bearing suitable characteristics as recommended for trout culture (Table 1). Utmost care was taken to maintain the stocking density of 10 kg/m^2 (mass) and water velocity of 50L/sec in all the raceways in order to rule out any possible impact of crowding stress. The sufficient availability of fish, culture facilities, and feasibility of distance from the research facility were also taken into consideration while selecting the study site.

2.2. Fish collection

For the long term assessment of fish blood chemistry, a monthly collection of fish was made from February 2019 to January 2020. For this purpose, a total of 444 apparently healthy fish which included 148 juveniles, 296 adults comprising of 148 male and 148 female fish were selected for the study, using exclusion criteria of external trauma/disease signals, including lesions on the skin, tail or fins. The health condition and sex of the fish were also confirmed afterwards, by following a standard necropsy procedure (Blazer et al., 2018). Intermittent fasting (12:12hr) fish were caught during the early morning hours at 8.00 to 9.00 (IST), before the scheduled feeding, from the raceways using a hand net. Afterwards, the fish were immediately transferred to the well-aerated containers containing water from the same source, following which, the collection of blood samples was made.

2.3. Blood sampling and processing

To reduce the handling stress, the fish within the containers were lightly anesthetized with sodium bicarbonate-buffered tricaine methanesulphate (MS-222 at 0.1 gL^{-1}) for 2–3 min, until the loss of coordination was visible (Topic Popovic et al., 2012).

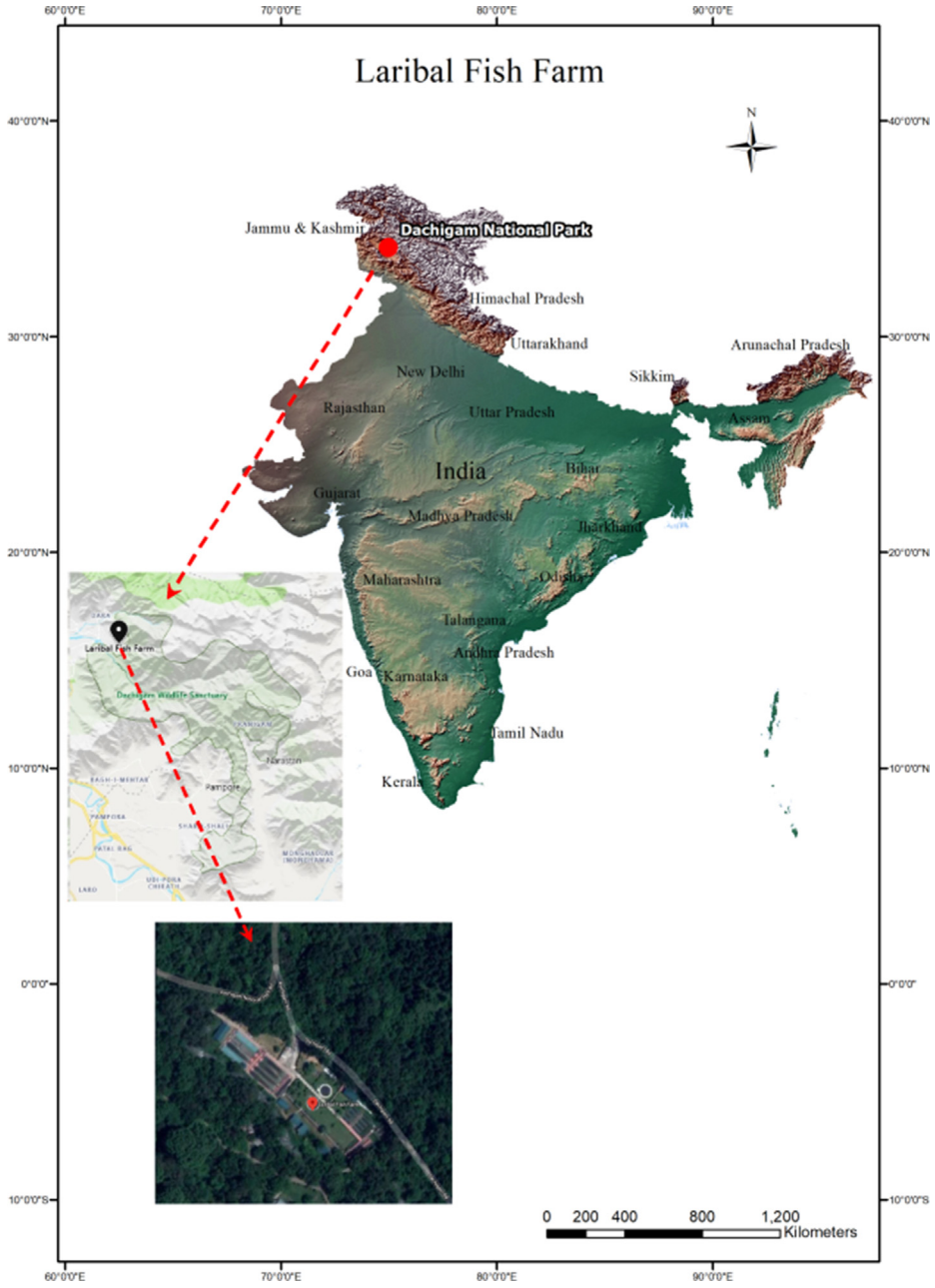

Fig. 1. Map showing the location and layout of the study site at Dachigam, Kashmir, India.

Table 1
Physico-chemical characteristics of the experimental raceway water.

Physico-chemical parameters	Raceway water	
	Range	Mean ± S.D.
Water temperature (°C)	8.2–14.2	14.23 ± 3.65
pH	7.22–7.86	7.84 ± 0.38
DO (mgL^{-1})	8.50–11.50	10.43 ± 1.58
Free CO_2 (mgL^{-1})	2.50–4.25	2.26 ± 0.75
Total alkalinity (mgL^{-1})	132.83–167.22	132.83 ± 2.48
Hardness (mgL^{-1})	187.34–195.31	191.73 ± 3.21

Afterwards, the blood was collected by puncture of the caudal tail vessels, using a 23-gauge needle (Dispovan, A896) attached to a 4 ml syringe (HMD, India). A part of the blood sample was transferred into 2 ml heparin-coated vials (Vactube Bio. Ltd. India) for the purpose of hematological analysis and the assessment of blood cell morphology. The remaining blood was poured into duplicates of 1.5 ml serum separating eppendorf tubes with no anticoagulant for performing the serum biochemistry assays. Blood samples were carried to the wet laboratory at Department of Zoology, University of Kashmir, Srinagar, where hemato-biochemical analysis was performed within an hour of the sampling event.

2.4. Hematological analysis

Hematological parameters investigated, included the assays for Hemoglobin concentration (Hb), hematocrit (Hct), total red blood cell count (RBC), total white blood cell count (WBC), thrombocyte count (TC) and derivation of erythrocyte indices. Hb was estimated by cyanomethemoglobin method (Lavanya et al., 2011) using Hemocor-D reagent (Coral Clinical Systems Ltd., India), Hct was determined by centrifuging the blood laden duplicate hematocrit

capillaries for each sample in a capillary centrifuge (REMI RM-12C, India) at 10,000*g* for 10 min at room temperature. An improved Neubauer hemocytometer was used to determine the total RBC, WBC + TC counts in blood mixed with Natt-Herrick's stain solution (Natt and Herrick, 1952). The number of cell counts was determined as described by (Pal et al., 2008; Parida et al., 2011). The total RBC count per $mm^3 = 200 \times 50 \times N \Rightarrow 10,000N$ (N = number of RBC counted, dilution factor = 200) and total WBC count per $mm^3 = \{(20 \times 1 \times L) \div (0.4)\}$cells $\Rightarrow 50 \times L$ (L = number of WBC counted dilution factor = 50). Erythrocyte indices such as mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) were calculated as per the standard formulae (Bain et al., 2006).

2.5. Blood smear preparation and Giemsa staining

Fresh blood smears were prepared simultaneously by expressing a drop of blood on a previously cleansed slide, placed on a horizontal surface. Afterwards, the edge of an another slide was touched to the periphery of the blood drop and uniformly pushed forward so that a thin blood smear is prepared. Duplicate slides of blood smears for each sample were prepared by fixing with methanol for 2–3 min and then left to air dry followed by staining with a 2:3 solution of Wright and Giemsa dye for nearly up to15 min (Loba Chemie, Mumbai, India). The whole procedure of smear preparation and staining was completed within 20 min. The stained samples were placed in a slide box until visualised under the light microscope.

2.6. Differential leucocyte count (DLC), morphology and morphometrics of peripheral blood cells

Prepared blood smears were examined under the oil lens at 100X magnification of light microscope (Leica DM 750) with inbuilt LAS X specifications that were used for quantification and morphometric measurement of different kinds of cells on a computer screen. Differential leucocyte and thrombocyte count was performed by counting up to 100 WBCs and then subtracting thrombocyte count from DLC+TC to yield the final WBC count. The photo imaging system was used to assess the cellular morphology and to measure cell dimensions such as cell length, cell diameter, nuclear length and nuclear diameter, whichever applicable for all different kinds of cells observed. A 10 μm scale was used for visualising and photo imaging of the cells.

2.7. Serum biochemical analysis

The eppendorf tubes (no anticoagulant) containing blood samples were centrifuged at 14,000*g* for 3 min within 30 min after blood harvest. (REMI RM-12C BL, India). Serum, rather than plasma was used for blood chemistry analysis to avoid possible interference of fibrinogen. A fixed volume (100 μL) pipette was used to dispense serum samples on the reagent rotor's sample port. The samples for each fish were analysed in duplicates for following analytes: Serum metabolites - blood glucose (GLU), total protein (TP), albumin (ALB), globulin (GLB), triglycerides (TG), total cholesterol (CHOL), creatinine (CRE), uric acid (UA) and blood urea nitrogen (BUN). Enzyme activities: alanine aminotransferase (ALT), aspartate aminotransferase (AST), alanine phosphatase (ALP) and creatine kinase (CK). The assessment of serum analytes was performed on Automatic Vet scan biochemistry analyser VS2 (Abaxis, USA). Electrolyte Plus reagent rotor was used for estimation of electrolytes (Cl, Na, K, Ca and P) on the same analyser. Bile acids (BA) and gamma-glutamyl transferase (GGT) could not be assessed as their values did not fall within the range of the analyser. The instrument's testing precision and accuracy were verified from studies conducted using the CLSI EP5-A2 (2004) and guidelines with modifications based on CLSI EP18-A (2002) for unit-use devices.

2.8. Biometrics and biological indices of the fish

Following sedation and blood sampling, biometrics (weight and length), hepatosomatic index (HSI) and Fulton's condition factor (K) were determined for each fish.

$$HSI = \text{Weight of liver} \div \text{Body weight} \times 100$$

$$K = \frac{W}{L^3} \times 100;$$ where "W" is body weight and "L" defines total length

2.9. Physico-chemical parameters of water

The physico-chemical characteristics such as water temperature, pH, dissolved oxygen, free carbon dioxide, total alkalinity and hardness were measured using standard methods (Baird et al., 2017).

2.10. Data management and statistical analysis

Data generated during the entire study was recorded in Microsoft Excel 2010. Hemato-biochemical RIs were determined according to the published guidelines of American Society for Veterinary Clinical Pathology (ASVCP) which strictly follows clinical laboratory and standards institute (CLSI) recommendations (Friedrichs et al., 2012). Reference value advisor, (BioStatistiques, National Veterinary School of Toulouse, Toulouse, France) (v.2.1, freeware) a set of macroinstructions for Microsoft Excel was used for analysing data. 95% RIs with 90% confidence intervals (CI) were calculated based on normality and symmetry of data distributions, outliers, and sample size. Normal distribution of data was assessed according to Anderson-Darling test using histograms and Q-Q plots, with $P < 0.05$ indicating normality. Moreover, the extreme values that potentially arise due to various factors, including the influence of seasons were screened, identified and later rejected as the outliers, using Tukey's interquartile range method/Dixon reed method. For each analyte, RI was determined by the robust method, when the distribution for the analyte was Gaussian, by the robust method with Box-Cox transformation, when the distribution was not Gaussian and given the sample size was as enough as recommended, the RI was determined using non-parametric method. The 90% confidence intervals of the limits of the nonparametric and parametric reference interval were determined using respective bootstrap methods. Significant ($P < 0.05$) differences in age specific variables that showed non normal distribution were assessed by Mann-Whiteney U test and for gender specific variables, all of which depicted normal distribution (Anderson-Darling test), were analysed by independent sample "t" test using the statistical program IBM. SPSS Statistics v. 20 (Windows 8.1., Microsoft Corporation. 2013). Principal component analysis (PCA) was performed using Minitab 18 statistical software.

3. Results

The biometric indices of the fish samples used in the study are shown in Table 2. Juvenile fish were small in size with an average weight of 131 g (±27) compared to adult fish, weighing 385.34 (±37.53). Biological indices such as hepatosomatic index (HSI) and Fulton's condition factor (K) were found to be in the optimal range indicating good health condition of fish.

Table 2
Descriptive statistics of the biometric variables and biological indices of rainbow trout, *O. mykiss*.

Age group	Juvenile		Adult	
Parameters	Mean ± S.D.	Range	Mean ± S.D.	Range
Weight (g)	131 ± 27.28	88.65–185.82	385.34 ± 37.53	260.0–433.20
Total length (cm)	22.23 ± 1.22	20.90–26.24	30.31 ± 1.52	27.20–33.50
HSI (%)	0.88 ± 0.25	0.82–1.05	1.05 ± 0.38	0.80–1.28
K	0.97 ± 0.23	0.82–1.07	1.16 ± 0.21	1.09–1.27

HSI, Hepatosomatic index; K, Fulton's condition factor.

3.1. Reference intervals for hematological analytes

Reference values along with the descriptive statistics of the hematological analytes are summarized in Tables 3 and 4. Among all the measured hematological variables, Hct, Hb, total red blood cell (RBC) count along with secondary red cell indices (MCH and MCH) were found to be significantly ($P < 0.05$) higher in adult fish in comparison to juveniles. Contrastingly total leukocyte (TLC) count particularly lymphocytes were significantly ($P < 0.05$) higher in juveniles than adult fish. Male and female fish differed significantly ($P < 0.05$) for Hb, total red blood cell (RBC) count and total leukocyte count (TLC). Mean variations in hematological parameters between life stages and sex of fish are depicted in Fig. 3A.

3.2. Cell morphology and morphometrics

Five distinct types of the blood cells in the peripheral blood of rainbow trout viz., erythrocytes, neutrophils (heterophils), lymphocytes, monocytes and thrombocytes were identified and distinguished. The morphological features of each cell type are shown in Fig. 2 (A - H) and their cellular morphometrics are given in Table 5.

3.3. Reference intervals for serum biochemical parameters

RIs along with descriptive statistics of the measured serum metabolites, enzyme and electrolyte concentrations are shown in Tables 6 and 7 respectively. Among all the measured parameters glucose, total protein, total cholesterol, triglycerides, creatinine varied significantly ($P < 0.05$) with respect to the life stage of fish. Total protein cholesterol along with uric acid also varied significantly ($P < 0.05$) with respect to sex.

3.4. Principal component analysis

Results of the principal component analysis, (PCA) when applied independently to each of the hematological and serum biochemical data sets, revealed that the corresponding principal components, PC1 and PC2 were able to explain about 80% and 92.7% of the variability existing within the respective data sets. Individual loadings contributed by each of the parameter to PC1 and PC2 are shown under Tables 8 and 9.

4. Discussion

4.1. RIs for hematological parameters

4.1.1. Erythrocyte parameters

Reference intervals for the hematological parameters form a fundamental diagnostics that help to predict the pathophysiological alterations in cultured fish species. In teleost fish, hematological parameters are largely described in terms of the erythrocyte and leukocyte parameters. Erythrocytes form the principal compo-

Table 3
Descriptive statistics for erythrocyte parameters of cultured rainbow trout, *O. mykiss*.

Analyte	Juvenile (N = 148)	Adult (Male) (N = 148)	Adult (Female) (N = 148)		Combined (N = 444)						
	Mean (±SD)			[γ] *P* value	Mean	Median	[α]Min.	[α]Max.	Reference Interval [‡](90% C.I.)	[*]D	[β]Method
Hb (g/dL)	9.30^{a} ± 0.50	11.35^{b} ± 0.68	11.13^{b} ± 0.80	0.021	10.60 ± 1.13	10.66	8.13	12.30	8.32–12.28 (8.26–8.14) (12. 26 –12.30)	g	R
RBC ($\times 10^{6}$/mm^{3})	1.16^{a} ± 0.08	1.92^{b} ± 0.16	1.73^{c} ± 0.20	0.039	1.60 ± 0.35	1.77	0.97	2.07	1.01–2.04 (1.0–1.03) (2.03–2.06)	g	R
Hct (%)	31.36^{a} 1.45	37.72^{b} ± 1.41	35.10^{b} ± 2.32	0.019	34.7 ± 3.2	35.0	29.0	40.0	29.0–40.0 (29.0–30.0) (39.0–40.0)	g	R
MCV(fL)	270.06^{b} ± 9.70	197.26^{b} ± 16.05	204.90^{b} ± 15.45	0.011	224.95 ± 35.51	198.92	187.82	29.97	188.78–288.46 (188.12–188.78) (287.13–290.0)	n	NP
MCH (pg)	80.03^{b} ± 1.74	59.23^{a} ± 3.49	64.91^{a} ± 4.29	0.022	68.03 ± 9.37	63.33	56.06	84.33	56.79–82.67 (56.60–57.11) (82.40 –83.04)	n	NP
MCHC (g/dL)	29.65^{a} ± 0.60	30.07^{a} ± 0.84	31.70^{a} ± 0.53	0.058	30.49 ± 1.11	30.41	27.47	32.89	28.14–32.76 (28.09–28.27) (32.40–32.82)	n	NP

Hb = hemoglobin; RBC = red blood cell; Hct = hematocrit; MCV = mean corpuscular volume; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration.
Values shown as mean (±SD).
Mean values sharing the same superscript across the rows are not significantly different ($P > 0.05$).
[γ] *P* value indicating significant difference of each variable between the subgroups determined by ANOVA or Kruskas Wallis H test on the respective data distributions (g. n), Mean values in rows sharing the same superscript are not significantly different ($P > 0.05$).
[α] Min; Max represent minimum and maximum data values observed.
[‡] Low. limit (90% CI); Upp. limit (90% CI) indicate the respective lower and upper bound limit around the reference interval with 90% confidence interval.
[*] Distribution of data: non Gaussian (n) or Gaussian (g).
[β] NP indicates non parametric method of transformation; R, robust method; RT, robust method post Box - Cox transformation.

Table 4
Descriptive statistics for leukocyte and thrombocyte count of cultured rainbow trout, *O. mykiss*.

Analyte	Juvenile (N=148)	Adult (Male) (N=148)	Adult (Female) (N=148)		Combined (N=444)						
	Mean (±SD)			*P* Value	Mean	Median	$^{\alpha}$Min.	$^{\alpha}$Max.	Reference Interval ‡(90% C.I.)	*D	$^{\beta}$Method
TLC (x 10^3/mm^3)	63.70^{a} ± 14.72	58.67^{c} ± 12.36	60.97^{b} ± 14.59	0.035	61.15 ± 14.05	60.00	31.32	90.75	34.13–85.0 (32.42–34.45) (84.75–90.23)	g	R
TLØ (x 10^3/mm^3)	49.32^{a} ± 9.97	44.21^{b} ± 9.38	45.81^{b} ± 9.37	0.041	46.44 ± 9.78	47.39	20.02	71.36	28.07–63.98 (27.16–28.56) (62.0–64.22)	g	R
SL (x 10^3/mm^3)	30.77^{a} ± 7.41	22.16^{b} ± 7.18	24.04^{b} ± 5.58	0.044	25.65 ± 7.69	23.52	14	44.8	14.86–42.00 (14.31–15.37) (40.88–43.12)	g	R
LL (x 10^3/mm^3)	18.55^{a} ± 6.64	22.00^{a} ± 9.44	21.89^{a} ± 7.07	0.062	20.82 ± 7.96	19.9	0	52.72	7.54–37.66 (6.62–8.01) (35.59–39.21)	g	R
NØ (x 10^3/mm^3)	11.10^{a} ± 3.21	10.80 ^{a} ± 5.04	11.01^{a} ± 5.16	0.057	10.99 ± 5.12	12.00	4.2	18.8	4.5–18.78 (4.5–4.5) (18.60–18.80)	g	R
MØ (x 10^3/mm^3)	3.2^{a} ± 1.78	3.63^{a} ± 1.69	4.02^{a} ± 1.48	0.066	3.62 ± 1.6	3.50	1.2	7.4	1.2–7.0 (1.2–1.2) (6.8–7.4)	g	R
TØ (x 10^3/mm^3)	42.29^{a} ± 13.92	45.71^{a} ± 13.87	45.38^{a} ± 13.84	0.055	44.5 ± 13.9	43.0	22	68	23.0–68.0 (22.3–22.3) (68–68)	g	R

TLC = total leukocyte count; TLØ = total lymphocytes; SL = small lymphocytes; LL = large lymphocytes; NØ = neutrophils; MØ = monocytes; TØ = thrombocytes.
γ,α,‡,*, β as described under Table 3.
Values shown as mean (±SD)
Mean values sharing the same superscript across the rows are not significantly different ($P > 0.05$).

nents of fish blood, both due their dominance in number and the vital functional role they play (Witeska, 2013). The number, shape and size of erythrocytes is linked to the variation in diagnostically important parameters, such as Hb, Hct, total red cell (RBC) count, and secondary erythrocyte indices which include MCV, MCH and MCHC. In the present study, all these parameters shall be described ahead as the erythrocyte parameters. The RIs for erythrocyte parameters have been described in different teleost fish species (Table 10). With regard to the work on rainbow trout raised under Himalayan aquaculture, the baseline reference values are being reported for the first time, with a view that RIs derived here will serve as a reference database for future studies on the cultured fish. Examination of Hct, RBC and WBC values is particularly recommended on a routine basis to monitor the health of fish stocks in the farms (Fazio, 2019). Hematocrit, (Hct) also referred to as packed cell volume is regarded as one of most reliable and relatively stable hematological diagnostic parameter in fish (Ahmed et al., 2020). Highly active fish are supposed to have more elevated Hct values than the less active ones (Campbell, 2015), however, generally it ranges between 20% and 45% in healthy fish (Hrubec and Smith, 2010). Our findings established, the Hct range for rainbow trout between 29% and 40% which falls within the above mentioned range. Hemoglobin (Hb) in teleost fish exists as a tetrameric protein-iron complex with multiple isoforms and varying levels that are tightly coupled to adaptive response of fish to the variable dissolved oxygen levels in its surroundings (De Souza and Bonilla-Rodriguez, 2007). In the current study, reference range for hemoglobin in *O. mykiss* was noted between 8.35 and 12.24 g/dl. These higher levels of hemoglobin concentration in Salmonids including *O. mykiss* can be attributed to their lower Hb-O_2 affinity in comparison to fish species like tench, carp or pike, which are relatively hypoxia tolerant (Nikinmaa, 2001). Hb levels for *O. mykiss* noted in the present study are comparable to the other teleost fish species like tilapia, shortnose sturgeon, yellow catfish, and striped bass (Table 10).

The RI for red cell count noted for cultured *O. mykiss* in the current study was found to range between 0.38 × 10^6 mm^{-3} to 1.30 × 10^6 mm^{-3}. Therefore, by comparison, *O. mykiss* falls under the category of fish, where the erythrocyte count is considerably very low (0.5–1.5 × 10^6 mm^3), as opposed to the group of fish, with extremely high counts (3.0–4.2 × 10^6 mm^3), as categorized by Soldatov, (2005). In fish species, for example, hybrid striped bass (*Morone chrysops* × *Morone saxatilis*), total RBC count is as high as 3 0.66–4.96 × 10^6 mm^{-3} (Hrubec et al., 1996). This vast difference between the red cell count among the categories of fish is often attributed to several factors including, the profound influence of the magnitude of their locomotor activity (Witeska, 2013), variable oxygen demand (Tran-Duy et al., 2008) and habitat (Ahmed and Sheikh, 2020). The red cell values outside the above mentioned range are often indicative of the stress caused by the factors like hypoxia, transportation, crowding etc (Fazio, 2019; Galagarza et al., 2017). Likewise, the lower levels of Hb together with Hct strongly imply fish anaemia (Campbell, 2015).

In addition to the primary blood parameters, the secondary red blood cell indices which include MCV, MCH and MCHC are also regarded as important diagnostic parameters to monitor fish health (Seibel et al., 2021). In fish, MCV, MCH and MCHC values normally range between 150 and 350 fL, 30 and 100 pg and 18 to 30%, respectively (Hrubec and Smith, 2010). Our results for these parameters are consistent with the above described range. The results noted for erythrocyte parameters in rainbow trout are comparable to many fish species listed under Table 10. An overall low MCV value, 224.95 fL noted for *O. mykiss* can be attributed to its higher oxygen demand and relatively small RBC size in comparison to shortnose sturgeon where mean MCV value is 400 fL (Knowles et al., 2006). MCV also depends on the degree of activeness of fish (Hrubec and Smith, 2010). Since *O. mykiss* are highly active, demand high oxygen and therefore have lower MCV values. Abnormal increase in MCV together with Hct, often denote response to various stressors, leading to the RBC swelling (McDonald and Milligan, 1992Hrubec and Smith, 2010Saravananet al., 2011), however the persistent increase is an indicative of an underlying physiological condition. Alike in most of the other fish species, MCV and MCH in *O. mykiss* depict a broad range reference interval (Fig. 3B.) due to the substantial variation in the size of RBC's of an individual fish. As the average red blood cell size in *O. mykiss* is relatively small (15.35 × 7.25 μm), therefore average MCH value for *O. mykiss* is fairly low than fishes that have larger RBC's such as Shortnose sturgeon (*Acipenser brevirostrum*), 16.03 × 10.7 L μm (Knowles et al., 2006). Moreover, with the maturation of RBC's, cells increase in size thereby, increasing MCV, MCH and MCHC values (Campbell, 2015). Our results depict that, overall RIs for erythrocyte parameters of cultured rainbow trout tighten up most probably in

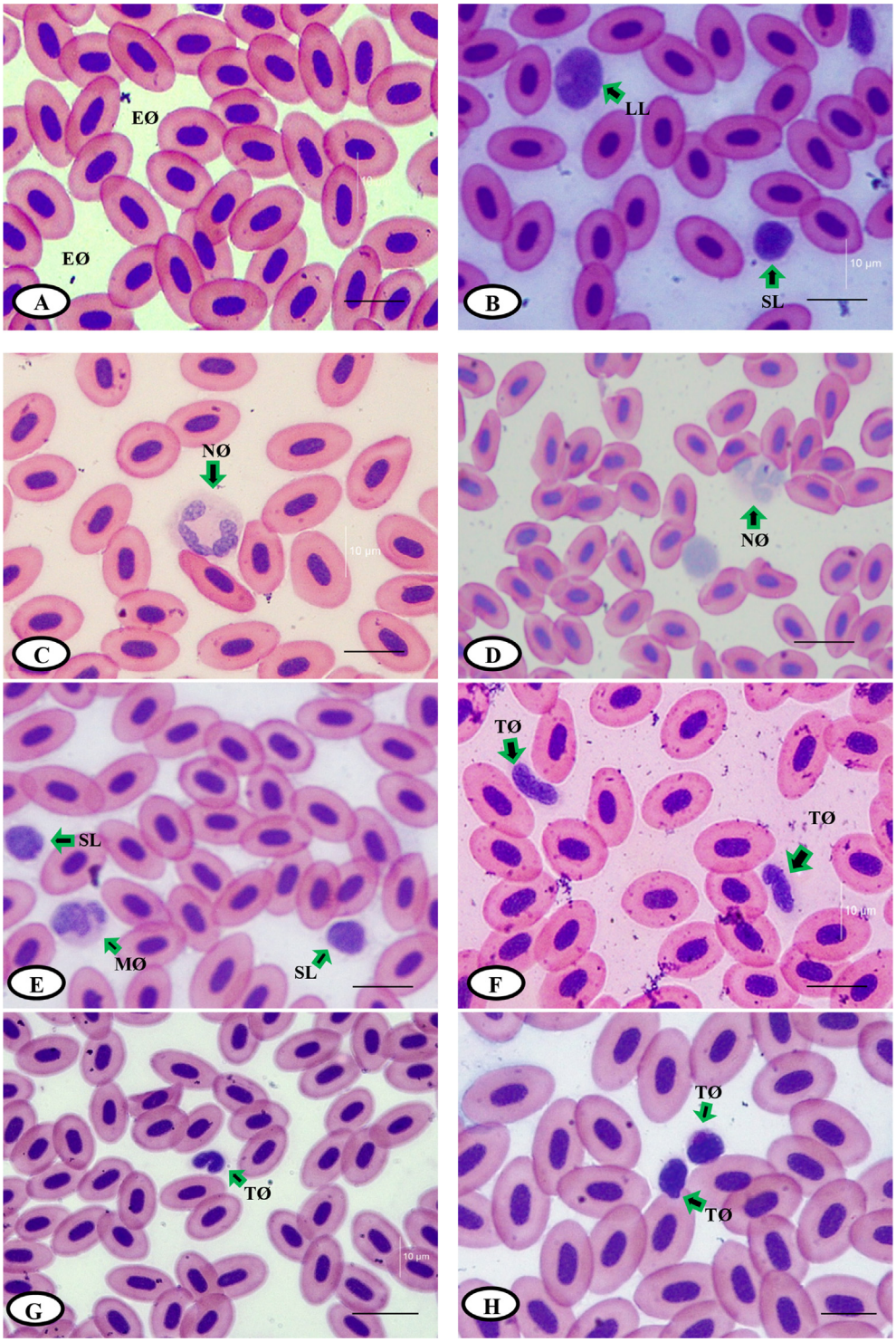

Fig. 2. Peripheral blood cells of rainbow trout, *O. mykiss* stained with Wright Giemsa. (A) Erythrocytes (EØ) depicting prominent eccentric nucleus surrounded by a clear cytoplasmic region. (B) A large lymphocyte (LL) and a small lymphocyte (SL) with nucleus surrounded by deep blue cytoplasm demarcated by a fine whitish rim at the cell periphery. (C) A neutrophil (NØ) with ribbon shaped, light blue coloured nucleus surrounded by pale lavender coloured cytoplasm. (D) A neutrophil (NØ) with heavy multi-lobed blue coloured nucleus surrounded with pale cytoplasm. (E) Monocyte (MØ) with indented nucleus and vacuolated cytoplasm; small lymphocytes (SL). (F) Thrombocytes (TØ), rod shaped with spindle-shaped or fusiform nucleus that conforms to the shape of the cell ending with transparent cytoplasmic protrusions. (G) Thrombocytes (TØ) with horse shoe shaped nucleus. (H) Thrombocytes (TØ), short statured with oval shaped nucleus and a protruding cytoplasmic end. Scale = 10 μm.

response to controlled aquaculture conditions, as opposed to the reports for different wild fish species (Table 10).

4.1.2. Leukocyte parameters

Trout leukocytes predominantly comprise of lymphocytes, neutrophils and monocytes (Blaxhall and Daisley, 1973). Unlike their mammalian counterparts, piscine literature bears inconsistencies with regard to nomenclature, classification and identification of various kinds of blood cells in fish, like neutrophils interchangeably are named as heterophils, moreover there are discrepancies in attributing the identification features to the fish basophils and eosinophils (Campbell, 2015). In this study, we successfully estab-

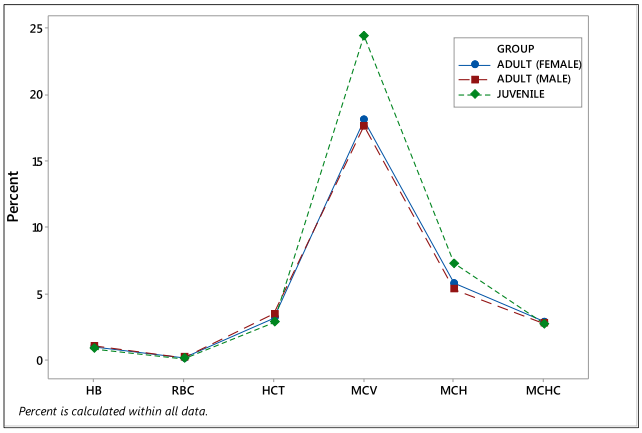

Fig. 3A. Line plot depicting percentage variation in erythrocyte indices between life stage and sex.

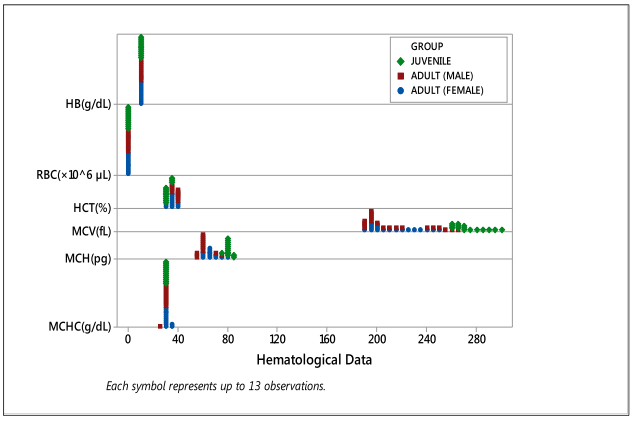

Fig. 3B. Dot plot showing distribution pattern of erythrocyte indices between life stages and sex.

lished RIs for the different cell types including lymphocytes, neutrophils, monocytes and thrombocytes for cultured *O. mykiss*. In the present, it was noted that total leukocyte (WBC) count for the cultured *O. mykiss* ranged between 28.00 and 75.00 ($\times 10^3$/mm^3). It has been previously found that, fish usually tend to have greater leukocyte count than the mammalian counterparts (Fang, 1992; Galagarza et al., 2017). Leukocyte parameters of the cultured *O. mykiss* in comparison with the other teleost fish species are listed under Table 11. It is evident from the comparison that RIs for leukocyte count of *O. mykiss* is in close approximation with shortnose sturgeon (*A. brevirostrum*) and striped bass (*Morone saxatalis*), however, the RI is fairly broad for species like hybrid striped bass, tilapia (*Oreochromis* Hybrid) and channel catfish (*Ictalurus punctatus*). Among different fish species, leukocytes predominantly have an immunological role to fight infection, therefore their absolute count serves as an important diagnostic tool to monitor fish health (Chen et al., 2021), although their count is affected by numerous factors including stress, age, sex, culture condition (Fazio, 2019). In this study, the DLC report revealed that lymphocytes (small and large) form the dominant cell type, accounting nearly for about > 50% of total leukocytes, followed by neutrophils (15–20%), a predominant granulocyte and the monocytes, which form about <5% of total leukocytes in the peripheral blood of the cultured *O. mykiss* (Fig. 4A). Generally, lymphocytes are abundantly found in the peripheral blood of fish in comparison to mammals, where they mostly reside in tissues, however under stress conditions, fish report lymphopenia, a condition with reduced leukocyte count (Valenzuela et al., 2008). Moreover, our study showed that peripheral blood of rainbow trout did not contain other types of granulocytes such as basophils and eosinophils.

In many fish species, the range for thrombocyte count is between 60 and 70 (x 10^3/mm^3) (Roberts and Ellis, 2001). However, as per the present study, thrombocyte numbers in rainbow trout ranged from 23.00 – 68.00 ($\times 10^3$/mm^3) which is consistent with studies on other freshwater teleost fish such as hybrid striped bass, 30.70–74.10 (x 10^3/mm^3); tilapia, 25.07–85.22 (x 10^3/mm^3) and striped catfish, 26.31–73.33 ($\times 10^3$/mm^3) (Hrubec et al, 1996; Hrubec et al, 2000; Galagarza et al., 2017).

4.1.3. Principal hematological components influenced by the life stage of fish

Hematological parameters in fish are as variable as in other animals, and the influencing factors include both the intrinsic as well as extrinsic (Clauss et al., 2008). Extrinsic factors, usually affect fish hemogram by inducing stress in response to conditions like poor water quality and quantity, inappropriate stocking and feeding, while as intrinsic factors such as age, sex, genotype, feeding habit, breeding etc. operate inherently. Hemoglobin concentration along with red blood cell (RBC) count notably varies in juvenile and adult fish primarily as a result of difference in metabolic and physical activity. Our results showed that levels of Hct, Hb and RBC along with secondary red blood cell indices (MCV, MCH) increase significantly ($P < 0.05$) in the adult fish which is well in corroboration with studies on the other fish species (Fazio et al., 2017; Karim et al., 2019; Fazio et al. 2020a). In the present study, the two principal components, (PC1 and PC2) of the hematological data versus life stage (juvenile and adult) were able to explain around 80% of the variation in the dataset (Fig. 4A). Large positive loadings of Hb, RBC and Hct show a strong correlation with PC1, however erythrocyte indices including MCV and MCH generate large positive loadings for PC2 (Table 8, Fig. 4B). This means that the PCA was able to efficiently distinguish between the life stages of rainbow trout, as the life stage-influenced hematological variables led to the separation of about 80% of data along PC1 and PC2 axis (Fig. 4A). Previously, Fazio et al. (2020a) has reported that the RBC levels increase significantly in elder striped bass, *M. saxatalis*, perhaps in response to the increase in metabolic activity and energy demand as the fish grows in weight. Moreover, the

Table 5
Morphometrics of peripheral blood cells in cultured rainbow trout, *O. mykiss*.

Morphometric parameter	Erythrocytes	Large lymphocytes	Small lymphocytes	Neutrophils	Monocytes	Thrombocytes	
						Long	Short
Cell length (µm)	15.35 ± 2.45	11.66 ± 2.12	8.12 ± 1.32	10.44 ± 1.23	10.63 ± 1.45	7.68 ± 1.21	3.63 ± 0.23
Cell width (µm)	7.25 ± 1.14	8.24 ± 1.36	7.20 ± 1.12	12.12 ± 1.28	13.21 ± 1.23	3.21 ± 0.11	3.18 ± 0.12
Nuclear length (µm)	6.6 ± 1.12	–	–	6.41 ± 0.56	7.24 ± 0.87	–	–
Nuclear width (µm)	3.45 ± 0.65	–	–	4.23 ± 0.45	5.32 ± 0.55	–	–

Values shown as mean (±SD).

Table 6
Descriptive statistics for serum metabolites of cultured rainbow trout, *O. mykiss*.

Analyte	**Juvenile** (N=148)	**Adult (Male)** (N=148)	**Adult (Female)** (N=148)		**Combined** (N=444)						
	Mean (±SD)			$^{\gamma}P$ value	Mean	Median	$^{\alpha}$Min.	$^{\alpha}$Max.	Reference Interval ‡(90% C.I.)	*D	$^{\beta}$Method
Glucose (mmolL^{-1})	2.12^{a} ± 0.12	2.55^{b} ± 0.09	2.30^{b} ± 0.09	0.012	2.32 ± 0.20	2.31	1.88	2.75	1.92–2.70 (1.91–1.94) (2.68–2.70)	g	R
T. protein (gdL^{-1})	3.17^{a} ± 0.10	3.71^{c} ± 0.14	3.50^{b} ± 0.10	0.019	3.49 ± 0.26	3.50	3.00	4.00	3.0–3.9 (3.0–3.10) (3.90–3.90)	g	R
Albumin (gdL^{-1})	1.36^{a} ± 0.09	1.50^{b} ± 0.10	1.44^{ab} ± 0.08	0.016	1.44 ± 0.11	1.40	1.10	1.60	1.20–1.60 (1.20–1.20) (1.60–1.60)	g	R
Globulin (gdL^{-1})	1.80^{a} ± 0.10	2.20^{b} ± 0.11	2.13^{b} ± 0.09	0.021	1.70 ± 0.20	2.10	0.20	2.50	1.70–2.40 (1.63–1.71) (2.37–2.45)	g	R
T. cholesterol (mmolL^{-1})	4.99^{a} ± 0.70	5.69^{b} ± 1.27	7.18^{c} ± 1.12	0.016	5.96 ± 1.4	5.85	1.4	9.05	4.09–8.83 (4.08–4.11) (8.33–9.03)	g	R
Triglycerides(mmolL^{-1})	2.65^{a} ± 0.27	3.76^{b} ± 0.42	3.84^{b} ± 0.31	0.011	3.42 ± 0.64	3.59	0.64	4.63	2.20–4.46 (2.19–2.24) (4.44–4.50)	n	RT
BUN(mmolL^{-1})	1.63^{a} ± 0.52	1.72^{a} ± 0.71	1.57^{a} ± 0.35	0.067	1.64 ± 0.55	1.64	0.55	5.60	0.56–2.82 (0.46–0.65) (2.46–2.96)	g	R
Creatinine(μmolL^{-1})	30.14^{a} ± 11.45	51.65^{b} ± 9.89	36.61^{ab} ± 12.16	0.009	39.45 ± 14.40	35.73	20.87	68.12	22.10–66.19 (22.10–22.10) (64.53–66.30)	g	R
T. Bilirubin(μmolL^{-1})	4.33^{a} ± 0.60	4.32^{a} ± 0.59	4.59^{a} ± 0.75	0.071	4.41 ± 0.66	4.28	2.35	5.81	3.39–5.78 (3.34–3.43) (5.68–5.81)	g	R
Uric acid(μmolL^{-1})	29.92^{ab} ± 6.63	36.48^{a} ± 8.10	34.08b ± 7.19	0.041	33.49 ± 7.79	32.88	14.28	52.85	18.45–49.52 (17.38–19.36) (48.30–50.52)	n	RT

γ,α,‡,*, β as described under Table 3.
Mean values sharing the same superscript across the rows are not significantly different ($P > 0.05$).

Table 7
Descriptive statistics for serum enzymes and electrolyte concentration of cultured rainbow trout, *O. mykiss*.

Analyte	**Juvenile** (N = 148)	**Adult (Male)** (N = 148)	**Adult (Female)** (N = 148)		**Combined** (N = 444)						
	Mean (±SD)			$^{\gamma}P$ value	Mean	Median	$^{\alpha}$Min.	$^{\alpha}$Max.	Reference Interval ‡(90% C.I.)	*D	$^{\beta}$Method
ALT (μkatL^{1})	0.17^{a} ± 0.06	0.27^{a} ± 0.04	0.24^{a} ± 0.05	0.063	0.23 ± 0.04	0.25	0.12	0.35	0.13–0.35 (0.12–0.13) (0.34–0.35)	n	NP
AST (μkatL^{1})	6.24^{a} ± 2.26	9.33^{a} ± 2.17	8.09^{a} ± 1.95	0.058	7.95 ± 2.10	7.90	3.69	11.97	3.91–11.88 (3.76–3.97) (11.82–11.96)	n	NP
ALP (μkatL^{1})	1.42^{a} ± 0.57	2.2^{a} ± 0.78	2.13^{a} ± 0.73	0.067	1.93 ± 0.63	1.66	0.75	3.81	1.10–3.42 (1.09–1.10) (3.36–3.43)	n	NP
CK (μkatL-^{1})	23.42^{a} ± 4.17	21.28^{a} ± 4.20	21.55^{a} ± 3.65	0.071	22.08 ± 0.38	22.57	12.63	30.14	14.37–29.14 (13.72–14.54) (28.49–29.64)	n	NP
Chloride(mmolL^{-1})	129.22^{a} ± 5.10	130.25^{a} ± 5.70	128.58^{a} ± 5.57	0.067	129.34 ± 6.32	129.50	113.34	145.43	117.85–139.55 (116.77–118.75) (139.32–142.10)	n	NP
Sodium(mmolL^{-1})	140.51^{a} ± 7.59	144.80^{a} ± 7.48	141.08^{a} ± 5.83	0.066	142.10 ± 7.32	141.0	130.0	160.0	127.20–156.10 (126.30–128.10) (155.2–157.20)	n	NP
Potassium(mmolL^{-1})	3.67^{a} ± 0.27	3.58^{a} ± 0.30	3.66^{a} ± 0.25	0.063	3.64 ± 0.48	3.70	3.00	4.20	3.09–4.23 (126.30–128.10) (4.21–4.29)	n	NP
Calcium(mmolL^{-1})	11.95^{a} ± 0.38	12.87^{a} ± 0.84	12.67^{a} ± 0.80	0.069	12.50 ± 0.64	12.50	11.30	14.30	11.40–14.10 (11.30–11.40) (14.10–14.20)	n	NP
Phosphorus(mmolL^{-1})	8.60^{a} ± 0.66	9.05^{a} ± 1.01	8.76^{a} ± 0.84	0.059	8.81 ± 1.02	8.70	7.60	12.10	7.55–10.97 (7.49–7.61) (10.73–11.22)	n	NP

ALT = alanine aminotransferase; (AST) = aspartate aminotransferase; ALP = alkaline phosphatase; CK = creatine kinase.
γ,α,‡,*, β as described under Table 3.
Mean values sharing the same superscript across the rows are not significantly different ($P > 0.05$).

Table 8
PCA matrix for hematological parameters of cultured rainbow trout (Juvenile versus Adult).

Variable	HB	RBC	HCT	MCV	MCH	MCHC	TLC	TLØ	SL	LL	NØ	MØ	TØ
PC1	**0.363**	**0.325**	**0.343**	−0.298	−0.28	0.223	0.27	0.186	0.134	0.096	0.286	0.325	0.327
PC2	−0.178	−0.281	−0.177	**0.33**	0.349	−0.098	**0.335**	0.291	**0.464**	−0.091	0.293	0.225	0.245
PC3	−0.061	−0.033	−0.111	−0.026	0.005	0.108	0.255	**0.513**	−0.095	**0.722**	−0.204	−0.2	−0.174

Principal components (PC1, PC2).

amplified RBC levels observed in older fish are often linked to the increased erythropoiesis (Svetina et al., 2002). Similar increase in RBC and Hb content has been reported in *Tenulosa ilisha* and *Mugil cephalus* (Jawad et al., 2004; Fazio et al., 2013). Increase in Hct levels can be explained by the significant rise in total RBC count, as they form the predominant cell species, and henceforth affect the Hct levels. Besides this, Hct levels also increase as the fish become more active (Galagarza et al., 2017). In the present study,

Table 9
PCA matrix for serum metabolites of cultured rainbow trout (Juvenile versus Adult).

Variable	GLU	TP	ALB	GLB	T.C	TRY	BUN	CRE	T.BIL.	U. ACID
PC1	**0.419**	**0.463**	0.285	0.43	**0.207**	**0.392**	−0.001	**0.309**	0.03	0.21
PC2	−0.189	−0.004	−0.317	0.169	**0.415**	0.241	−0.332	−0.102	0.623	−0.31

Principal components (PC1, PC2).

Table 10
Erythrocyte parameters of the cultured rainbow trout in comparison to other teleost fish.

Species	Hct (%)	Hb (g/dL)	RBC ($\times 10^6$/mm^3)	MCV(fL)	MCH (pg)	MCHC (g/dL)	Reference
Striped bass (hybrid)	23–47	8–12	3.66–4.96	81–106	19.6–26.4	22–30	Hrubec et al., 1996
Tilapia	27–37	7.0–9.8	1.91–2.83	115–183	28.3–42.3	22–29	Hrubec et al., 2000
Shortnose sturgeon	26–46	5.7–8.7	0.65–1.09	307–520	65.9–107.1	15–30	Knowles et al., 2006
Channel catfish	40.00	–	2.44	–	–	–	Tavares-Dias and De-Moraes, 2007
Yellow catfish	21.40–55.61	7.2–9.9	1.66–2.43	–	–	–	Prasad and Charles, 2010
Senegalese sole	9.41–26.67	2.01–6.02	1.10–2.34	82.49–165.90	17.86–81.90	13.89–27.34	Peres et al., 2015
Striped bass	25.30–46.30	8.70–12.50	2.28–3.93	89–151	31.90–47.20	25.90–37.80	Fazio et al., 2020
Rainbow trout	29–40	8.35–12.24	1.01–2.04	189–288	56.81–82.68	28.14 –32.76	Present study

Table 11
Leukocyte and thrombocyte parameters of the cultured rainbow trout in comparison to other teleost fish.

Parameters	Striped bass (hybrid)	*Tilapia	*Shortnose sturgeon	Channel catfish	Senegalese sole	Striped catfish	Rainbow trout
Total Leukocytes (x 10^3/mm^3)	32.60–115.10	21.56–154.69	28.38–90.79	8.90–124.00	66.1–206.0	36.30–94.29	34.13–85.0
Neutrophils/ Heterophils (x 10^3/mm^3)	4. 00–3.50	0.56–9.87	3.76–33.60	4.5–86.8	2.0–9.3	4.50–18.29	4.50–18.78
Total lymphocytes (x 10^3/ mm^3)	22.50–115.10	–	–	1.4–23.6	25.0–113.0	18.99–59.99	28.07–63.98
Small lymphocytes (x 10^3/ mm^3)	–	6.78–136.39	9.06–56.66	–	–	13.76–51.49	14.86–42.0
Large lymphocytes (x 10^3/ mm^3)	–	2.85–30.83	2.12–10.44	–	–	0.71–21.20	7.54–37.66
Monocytes (x 10^3/mm^3)	1.50–7.50	0.40–4.29	0–7.14	0.70–14.70	50.0–65.0	0–7.55	1.20–7.0
Thrombocytes (x 10^3/mm^3)	30.70–74.10	25.07–85.22	32.20–122.18	–	29.0–66.0	26.32–73.33	23.0–68.0
References	Hrubec et al., 1996	Hrubec et al., 2000	Knowles et al., 2006	Tavares-Dias and De-Moraes, 2007	Peres et al., 2015	Galagarza et al., 2017	Present study

*Unit conversion made for the convenience of comparison.

the total leukocyte count especially the lymphocyte count of juveniles was found to be significantly ($P < 0.05$) higher in comparison to the adult fish. Similar results have been reported by other workers (Blaxhall, 1972; Hrubec et al., 2001). The PCA analysis, however recorded, TLC as the major variable that contributed largely to the PC3 (eigen value > 1) (Table 8), therefore, collectively explaining about 93% of the existing variation in rainbow trout hematology. Fig. 5A indicates that percentage of total lymphocyte count (TØ), small lymphocyte count (SL) and large lymphocyte count (LL) varies to a greater extent between juvenile and adult fish, (both sexes) therefore, they are the major contributors that significantly alter total leukocyte count (TLC). However, other types of leukocytes such as neutrophils (NØ) and monocyte (MØ) count remain fairly constant, therefore RIs for these components depict symmetric distribution pattern across all the categories in contrast to RIs for (TØ), (SL) and (LL) (Fig. 5B).

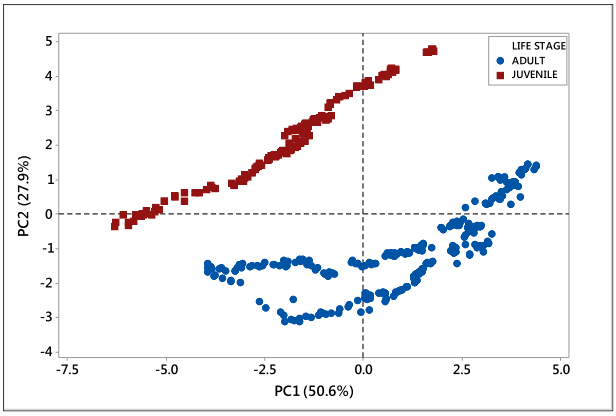

Fig.4A. Score plot of hematological parameters for juvenile and adult fish.

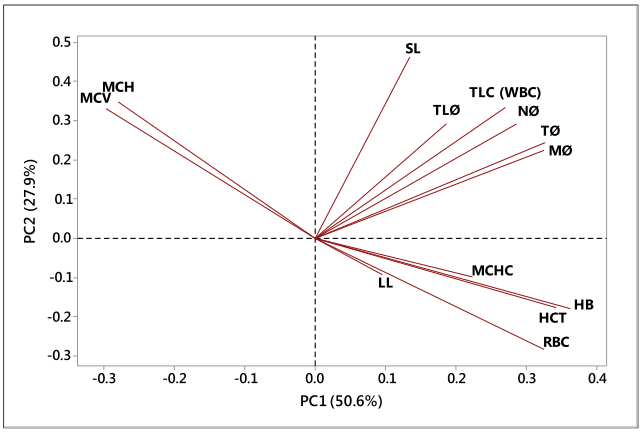

Fig. 4B. Loading plot of hematological parameters for juvenile and adult fish.

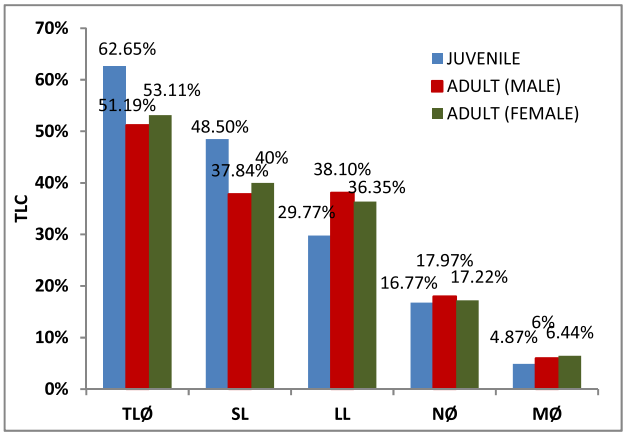

Fig. 5A. Variation in percentage composition of leukocytes between life stages and sex.

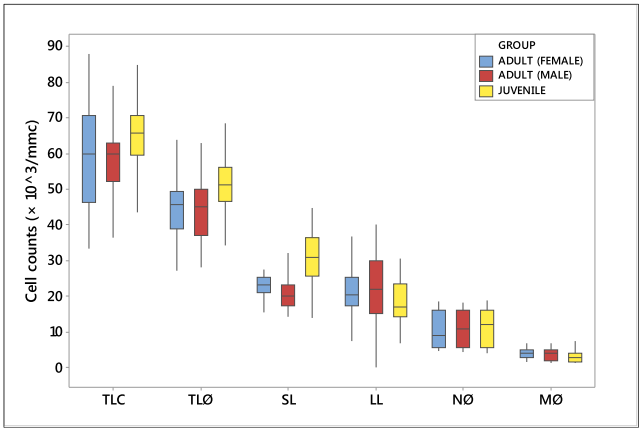

Fig.5B. Box plots depicting reference intervals for TLC and DLC between life stages and sex.

4.1.4. Hematological variations with respect to sex

With regard to the variation of hematological parameters with respect to the sex of cultured rainbow trout, we found that except for RBC and TLC, no significant difference ($P > 0.05$) was noted for all the other measured parameters. By comparison of mean values using ANOVA, it was noted that RBC were significantly ($P < 0.05$) higher in males, (Table 3) whereas TLC were significantly ($P < 0.05$) higher in female fish (Table 4). Higher red cell count in male fish may be attributed to its relatively higher metabolic rate that up regulates the erythropoietin activity, the major hormone that induces the red cell synthesis in fish (Zakęś et al., 2016; Sharma et al., 2017; Jan et al., 2021). Contrastingly, the higher TLC in female cultured *O. mykiss* indicates that females are immunologically more robust than males due to their genetic makeup that allows them to combat infections more efficiently. Findings for other freshwater teleost fish also support similar kind of variations between the two sexes (Witeska, 2013; Ahmed et al., 2019; Jan et al., 2021).

5. Blood cell morphology and morphometrics

Piscine literature historically classifies the fish blood cells as erythrocytes, leukocytes and thrombocytes. Studies on teleost fish generally categorise piscine leukocytes in line with their classification in mammalian counterparts as lymphocytes, neutrophils or heterophils and monocytes (Campbell, 2015).

Mature erythrocytes of the *O. mykiss* were found to be ellipsoidal in shape with smooth outer boundary, eccentric nucleus that occupies the major portion of the cell and is elongated in the direction that marks the larger axis of the cell, the cytoplasm appears pinkish without any surface protrusions (Fig. 2A). The erythrocyte size of fish bears an adaptive value and is inversely related to the metabolic rate (Dal'Bó et al., 2015). The major and the minor axis measurements of the rainbow trout erythrocyte nearly yield a value of 15.35 × 7.25 μm which is in approximation with brown trout, *Salmo trutta*, 11 × 8 μm and grass carp, 12.31 × 8.37 μm (Blaxhall and Daisley, 1973; Chen et al., 2019). Erythrocyte size increases in teleost fish during the maturation in contrast to mammals and birds where the size decreases (Dal'Bó et al., 2015). Change in erythrocyte morphometrics is also seen during the natural sex reversal in eel, *Monopterus albus* (Chen et al., 2021). Similarly, the nuclear length and width of rainbow trout erythrocyte observed here, 6.61 × 3.45 μm is in corroboration with studies on other teleost fish such as blunt snout bream, 6.38 × 3.63 μm, grass carp, 5.17 × 3.59 μm (Chen et al., 2019). As per Blaxhall and Daisley (1973), the lymphocyte populations are dynamic because, the large cells act as the precursors of the smaller lymphocytes. Although smaller cells exist in abundance than the larger, however there seems to be no functional difference between the two (Barber et al., 1981; Chen et al., 2019). In our study, the smaller lymphocytes measured 8.12 × 7.20 μm in comparison to large ones, 11.66 × 8.24 μm. In rainbow trout, smaller lymphocytes appear as round cells, however most of them are not completely round in shape, the nucleus occupies the most of the space within the cell leaving a whitish rim of cytoplasm at its periphery (Fig. 2E). Light microscopy could not ascertain the nuclear and cytoplasmic extents of lymphocytes in terms of morphometric values, however apparently they have very high nuclear to cytoplasmic ratio than erythrocytes. Large lymphocytes of rainbow trout appear morphologically similar to those reported by Galagarza et al (2017) in cultured striped catfish, *Pangasius hypothalamus* and Hrubec et al., (2000) in cultured tilapia, *Oreochromis* hybrid. Among the other types of leukocytes, neutrophils were found to be more abundant than the monocytes in the peripheral blood of the cultured *O. mykiss*. Neutrophils are roughly round shaped cells with polymorphic nucleus staining light to dark blue surrounded by pale lavender cytoplasm, the nucleus is variously shaped from ribbon like band (Fig. 2C) to a sharply indented and segmented structure (Fig. 2D) measuring about 10.44 × 12.12 μm in size. Neutrophil nucleus assumes different shapes in other teleost fish such as brown trout, *Salmo trutta* where it exists as ribbon or sac like form and roughly the overall cell size measures about 9.1 μm (Blaxhall and Daisley, 1973). Overall nuclear size of rainbow trout neutrophil measures about 6.41 × 4.23 μm which is comparable to that reported for yellow catfish, *Pelteobagrus fulvidraco*, 6.97 × 5.35 μm and blunt snout bream, *Megalobrama amblycephala* 6.46 × 4.70 μm (Chen et al., 2019). On the other hand monocytes in rainbow trout are a least populous group of mononuclear phagocytes with partially lobed deep blue stained nucleus that lies at the periphery of the cell, leaving the rest of space for the translucent to pale bluish abundant cytoplasm which is actively involved in the phagocytic function, as indicated by the presence of dense vacuoles (Fig. 2E). The monocytes measured approximately 10.63 × 13.21 μm with nuclear dimensions of 7.24 × 5.32 μm marking a largest cell in the leukocyte family. Presence of monocytes have been also reported in the peripheral blood of the other fish such as grass carp, yellow catfish, blunt snout bream (Chen et al., 2019), striped catfish (Galagarza et al., 2017), tilapia hybrid (Hrubec et al., 2000) and shortnose sturgeon (Knowles et al., 2006).

Teleost thrombocytes, unlike their avian counterparts function exclusively in clotting process without being involved in active phagocytosis (Roberts and Ellis, 2001). Thrombocytes in rainbow trout vary between fusiform or spindle, kidney and roughly oval to circular in shape (Fig. 2F-H). The shape variation in the teleost thrombocytes is often linked to the degree of their maturity and activeness. Like in most of the other teleost species, spindle-shaped thrombocytes in rainbow trout, also appear to be the most reactive forms which exist in clusters with one pole of each cell often drawn out into a point, surrounded by a small amount of cytoplasm (Fig. 2F, H). Longer thrombocytes in rainbow trout measure approximately 8.12 × 3.21 μm in comparison to shorter cells measuring 3.63 × 3.18 μm. The morphological features of rainbow trout thrombocytes closely resemble those of channel catfish, *I. punctatus* and common carp, *Cyprinus carpio* (Tripathi et al., 2004; Tavares-Dias and De-Moraes, 2007).

6. RIs for serum biochemical parameters

In the present study, RIs for different serum biochemical attributes of the cultured rainbow trout were also determined. Serum glucose is regarded as an exceptional indicator of the fish health due to its rapid response to the various kinds of stressors (Hille, 1982; Polakof et al., 2011). Our results showed that fasting serum glucose levels are as low as 1.29–2.70 mmolL^{-1} in *O. mykiss*. Although, after feeding, serum glucose levels increase transiently beyond the above observed range because the glycaemic changes in trout serum are linked to the type and amount of carbohydrate in food (Polakof et al., 2012). Several teleost fish including, *O. mykiss* can tolerate extremely low levels of the serum glucose without suffering any sort of neural damage since they have very high levels of both the glucose transporter (GLUT 1) and glycogen in their neural tissue (Mommsen and Plisetskaya, 1991; Liang et al., 2020). Defining the RI for serum glucose is critically important to diagnose the stress levels in fish. It is well documented that stress induces epinephrine mediated hyperglycaemia by releasing glucose from the liver reservoirs that transiently doubles the serum glucose concentration in many teleost fish (Weber and Shanghavi, 2000; Hoffmayer et al., 2012). Additionally, blood glucose levels in trout seem to be far greatly sensitive to the influence of the variety of physiological and environmental conditions (Polakof et al., 2012). Findings on serum glucose levels in rainbow trout (Manera and Britti, 2006; Kopp et al 2011) and other freshwater fishes (Satheeshkumar et al., 2012; Ahmed and Sheikh, 2019) seem to appropriate our study.

The serum concentration of total protein that basically includes albumin and globulin fractions exhibit a normal distribution with relatively lower S.D in comparison to the most of other serum metabolites (Table 6). Invariable serum protein levels for *O. mykiss* are also reported by many other researchers, who attribute it to the minimal influence of exogenous factors on serum protein levels. (McCarthy et al., 1973; Hille, 1982; Manera and Britti, 2006). The RIs for total protein and its components differ from the previous reports on *O. mykiss* (McCarthy et al., 1973; Hille, 1982; Manera and Britti, 2006). This is most probably due to its dependence on the endogenous physiological factors which include body size and gender (Fazio et al., 2020a). In the present study, we found that the total serum protein of rainbow trout, RI: 3.0–3.9 gdL^{-1} had relatively higher globulin concentration, 1.70 (±0.20) gdL^{-1} than albumin 1.44(±0.11) gdL^{-1}. The albumin levels are found to be higher in fresh water fish than in marine counterparts (Morro et al., 2020) and are influenced by the degree of species natural mobility, season, stage of gonad maturity, and other factors (Andreeva, 2010). Fish immune function is largely governed by globulins, so the increase or decrease in this component has a clinical relevance (Peres et al., 2015). Observations of serum protein levels noted here are very similar to those reported previously for rainbow trout (Manera and Britti, 2006) and other freshwater fish species (Andrews et al., 2009; Di Marco et al., 2011).

Contrary to the sugar derived energy sources, serum lipids, particularly, cholesterol and triglycerides which represent fat origin energy substrates are more vital components required in meeting the energy demand in rainbow trout (Cho and Cowey, 1991; Hardy, 2002). Both total cholesterol and triglycerides followed normal distribution, therefore, RIs were computed using robust technique. Although the upper and lower boundaries of RIs for total cholesterol, RI: 4.09–8.83 mmolL^{-1} and triglycerides, RI: 2.20–4.46 mmolL^{-1} differ from the findings of Manera and Britti (2006); Ahmed and Sheikh (2019); Fazio et al. (2020); Reshma et al. (2020), however median values especially for total cholesterol, 5.85 mmolL^{-1} correspond to the above reported studies on rainbow trout and other fish species. The discrepancy in RIs of the lipid components is due to the differential requirement of lipids during various growth stages of fish (Jobling et al., 1998; Fazio et al., 2020a: Hrubec et al., 2001). Moreover serum lipid levels are tightly coupled to the nutritional state and dietary differences in fish (Peres et al., 2015).

With regard to the products of nitrogen metabolism, particularly, serum urea nitrogen conventionally called blood urea nitrogen (BUN) and creatinine, the RIs were noted in the range between 0.56 and 2.82 mmolL^{-1} and 22.10–66.19 μmolL^{-1} respectively. Both the variables seem to be influenced by multiple factors, primarily the differential availability and uptake of dietary protein. BUN values display a similar range in other freshwater fish (Owolabi, 2011). Usually serum creatinine concentrations are low in fishes and are a function of fish muscle dynamics and creatine production with physiological adaptations tending to fluctuate the serum creatinine levels (Knowles et al., 2006; Borchel et al., 2014). The upper limit RI for creatinine noted in the present study is slightly above the range as per Manera and Britti, 2006 and considerably below than upper limit assessed for cultured tilapia (Hrubec et al., 2000). The differences in the levels of blood urea nitrogen and creatinine may indicate dietary, rather than pathological differences (Ellsaesser and Clem, 1987; Wilkie, 2002).

Moreover, the two other serum biochemical attributes, total bilirubin and uric acid which are relatively under examined in rainbow trout were determined in this study. Total bilirubin, RI: 3.39–5.78 μmolL^{-1} was found to have relatively range higher concentrations than uric acid, RI: 18.45–49.52 μmolL^{-1} indicating wider RI for the later. The lower concentrations of total bilirubin in fish as reported in this study have been attributed to the lower activity of biliverdin reductase in fish compared to mammals (Fang and Lai, 1987). Upon applying standard unit conversions, the mean values and range for total bilirubin concentration in our study largely agreed with some earlier published work other fish species (Hrubec et al., 2000; Borges et al., 2004; Asadi et al., 2006; Zhou et al., 2009). Uric acid which is one of the main water-soluble antioxidants in human and fish blood plasma (Mochnik et al., 1994; Xue et al., 1998) was found to be present in very low concentrations in trout serum hence measured as micromoles per litre (μmolL^{-1}). RIs for uric acid in trout serum are being reported for the first time hence our study brings new information about trout blood chemistry. The mean values for uric acid in the current study roughly correspond to Cakici and Aydin (2006) who reported impact of pollution on different serum analytes including uric acid in the rainbow trout. Clinical relevance of serum uric acid concentrations are indicated in studies investigating impacts of toxicity on fish blood chemistry (Abdel-Tawwab et al., 2013; Mutlu et al., 2015), which imply the need to further the RI studies in trout.

Estimation of RIs for alanine aminotransferase, ALT (0.13–0.34) μkatL^{-1}; aspartate aminotransferase, AST (3.91–11.88) μkatL^{-1};

alkaline phosphatase, ALP (1.10–3.42) μkatL^{-1} and creatine kinase, CK (14.37–29.14) μkatL^{-1} were performed by direct non parametric method (NP), as all these parameters overall assumed a non-guassian (n) type of distribution (Table 7). Also, no significant ($P > 0.05$) difference was found in any of enzyme activities with respect to life stage or sex of fish. ALT, AST, ALP, and CK, are important non-specific plasma enzymes because they also exist in different organs like gills, liver, kidneys and heart of fish where their activity possess a clinical relevance to specify any organ dysfunction (Wagner and Congleton, 2004; Shahsavani et al., 2010). Moreover, enzyme activities in rainbow trout and other fish also happen to be highly responsive to environmental temperature and spawning time (Hrubec et al., 1997; Celik, 2004). Our results for enzyme activities differ from those reported by Manera and Britti, (2006) and Kopp et al. (2011).

Electrolytes are the best understood blood analytes and are regarded as reliable indicators of osmoregulation in fish (Galagarza et al., 2017). Like in most of the other animal species including fish, the concentration of sodium were noted to be slightly higher than that of chloride (McDonald and Milligan, 1992). RIs for potassium in our study, 3.09–4.23 mmolL^{-1} approximated with the normal range for other fresh water fish, 2 to 4 mmolL^{-1}. RIs depicted in the current study for calcium, 11.40–14.20 mmolL^{-}1 and inorganic phosphorus, 7.60–11.50 mmolL^{-1} are comparable to that of cultured tilapia (Hrubec et al., 2000). Serum calcium levels are regulated by total serum protein levels, water alkalinity and stress conditions.

6.1. Principal serum biochemical components influenced by the life stage of fish

In the present study, principal component analysis for the serum chemistry data performed for two different life stages (juvenile and adult) of cultured rainbow trout was clearly able to differentiate between these groups. Nearly, all the data points behaved as distinct non-overlapping groups along the PC1 component (Fig. 6A). PC1 alone explained about 76.4% of the variation within the data with largest loadings made by total serum protein (0.463), followed by glucose (0.419), triglycerides (0.392) creatinine (0.309) and total cholesterol (0.207) (Fig. 6B). Loadings for all these parameters fairly decreased in PC2 (Table 9), thereby, they can be used to predict or classify the fish on the basis of its life stage. Contrastingly, PC2 just explains about 16.3% of the variation in the analysed dataset with large positive loadings for total cholesterol (0.415) that fairly decreased in PC1. In contrast to the PCA of hematological variables, where, nearly 80% of data variationwere explained by PC1 and PC2, 92.7% of the variation within the serum chemistry dataset was explained by PC1 and PC2, which

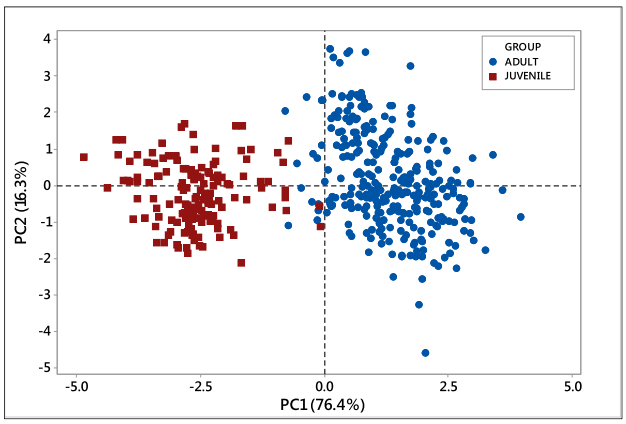

Fig. 6A. Score plot of serum biochemical parameters for juvenile and adult fish.

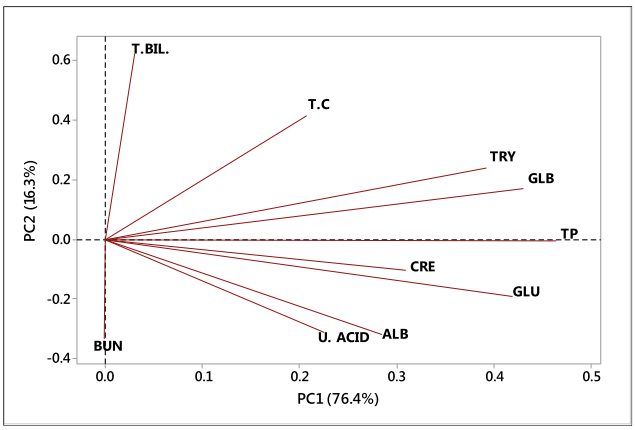

Fig. 6B. Loading plot of serum biochemical parameters for juvenile and adult fish.

marks the serum components as more suitable indices to distinguish between fish life stages. Parameters including glucose, total protein, total cholesterol, triglycerides and creatinine were found to significantly ($P < 0.05$) different between the juvenile and adult fish. All these variables increased significantly ($P < 0.05$) and were found to be higher in adult compared to the juvenile fish (Table 6). Principal elucidations that reason these differences include the differential metabolic rate, dietary requirements and mobility pattern among the juvenile and the adult fish (Hrubec et al., 2001; Fazio et al., 2020a). The parameters including glucose, total protein, total cholesterol and triglycerides have been found to be strongly correlated with the biometric indices in striped bass, *Morone saxatilis*, (Fazio et al., 2020a) revealing a strong agreement with the results obtained here. The increase in blood glucose and total cholesterol with an increase in fish size that primarily happens with the increasing age also confirms previous reports on rainbow trout and other freshwater fish (Hille, 1982; Hrubec et al., 2001; Fazio et al., 2020a). Total protein which constitutes albumin that acts in the transportation of steroid hormones (Shahsavani et al., 2010) is expected to increase with age and gonad maturation as a normal physiological process. Similar results of increase in total serum protein have been reported for striped bass (Hrubec et al., 2001) where serum globulin fraction increases to greater extent than the albumin fraction thereby altering the serum protein levels significantly.

6.2. Serum biochemical variations with respect to sex

The serum biochemistry dataset (adult male vs adult female) could not be classified using PCA technique therefore we only considered ANOVA results to monitor the mean differences of variables between the different categories. Interestingly, only three variables including total protein, total cholesterol and uric acid among the twenty variables tested on independent samples were found to differ significantly ($P < 0.05$) between the male and female fish. This signifies that the life stage influences the serum biochemical parameters of cultured rainbow trout more profoundly than its sex. Except for total cholesterol, the mean values for the other two variables including total protein and uric acid were found to be higher in males compared to females (Table 6). Higher serum cholesterol in female fish is the requirement for proper gonad maturation and also meant for the preparing the sexually mature female fish for breeding and spawning phases (Chatzifotis et al., 2004). Higher value of total protein in males is perhaps required to impart greater mobility to male fish. Changes in total serum protein between male and female fish have also been reported in tench, *Tinca. tinca* and grayling *Thymallus thymallus* (Svoboda et al., 2001). Significantly higher concentrations of uric acid found

in male fish are due to the need for accumulation of uric acid by mature male fish in seminal plasma necessary for the antioxidant defence of fish spermatozoa (Ciereszko et al., 1999).

7. Conclusion

Results of this study propose that principal serum biochemical components such as glucose, total protein, total cholesterol, triglycerides and creatinine are far more reflective of the life stage of fish than the hematological components such as Hb, Hct, RBC, MCV and MCH, hence they can be regarded as best life stage specific health indices of fish stocks. The hemato-biochemical profiling of rainbow trout reported here will certainly improve the application of clinical chemistry in fish medicine and invigorate management strategies for aquaculture practices.

8. Data availability

The authors declare that the datasets used in this study will be made available on reasonable request.

Ethical approval

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. The fish were handled in accordance with the guidelines approved by the institutional animal ethics committee (IAEC) prescribed by committee for the purpose of control and supervision of experiments on animals (CPCSEA) under R. No. 801/GO/Re/S/2003/CPCSEA.

Author contribution

Naveed Nabi carried out the experimental work, statistical analysis of the data and drafted the manuscript. Imtiaz Ahmed conceived, designed, reviewed the manuscript and supervised the study. Gohar Bilal Wani also supervised the drafting of the manuscript and aided in the arrangement of specimens for experimental work. All authors read and gave approval of the final manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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