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Physiological Responses of Kalibaus (*Labeo calbasu*) to Temperature Changes: Metabolic, Haemato-Biochemical, Hormonal and Immune Effects

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Abstract

A global interest in the cultivation of Kalibaus (*Labeo calbasu*) has emerged due to decreasing natural stocks and a consistent rise in market value and demand. Given these concerns, understanding the species' physiological responses to environmental changes is crucial. The present research aimed to assess the effect of varying environmental temperatures on metabolism, haemato-biochemical indices, hormonal concentrations and immune responses in *L. calbasu*. This study was conducted in triplicate using 100 L glass aquariums at four different temperatures: 22, 26, 30, and 34 °C. The highest weight and length gain were observed at 30 °C, while the lowest occurred at 22 °C. Notably, the best feed conversion ratio (FCR) of 1.51 ± 0.03 was also recorded at 30 °C. Although haematological and biochemical parameters remained within normal ranges, they varied with temperature changes. Indicators of cold and heat stress were evident through lower hematocrit levels and higher white blood cell (WBC) counts. Biochemical indicators such as serum albumin (1.84 ± 0.05 g dL^{-1}), serum globulin (1.64 ± 0.06 gdL^{-1}), HCO_3 (30.93 ± 0.62), Na^+ (115.60 ± 3.72 $mmolL^{-1}$), alkaline phosphatase (93.33 ± 9.39 AP, IUL^{-1}), and AST/SGOT (21.00 ± 4.55 UL^{-1}) were significantly higher at 30 °C. Regarding hormonal responses, peak levels of growth hormone (GH), triiodothyronine (T3) (1.44 ± 0.07 $ngmL^{-1}$), and thyroxine (T4) were recorded at 30 °C. Meanwhile, serum cortisol (1.62 ± 0.06 μgdL^{-1}) and adrenocorticotropic hormone (ACTH) (18.01 ± 3.26 $pgmL^{-1}$) were highest at 34 °C. Immune responses were strongest between 26 and 30 °C. In conclusion, the results suggest that *L. calbasu* should ideally be cultured between 26 and 30 °C for optimum growth and health, making it ideal for commercial farming.

Keywords: Kalibaus; temperature; metabolism; haemato-biochemical; hormone; immunoglobulin

Key Contribution: We conducted a comprehensive analysis of the temperature effects on the metabolism; haemato-biochemical parameters; hormonal concentrations; and immunity of *Labeo calbasu* under controlled conditions. Our results highlight that both the increase and

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decrease in temperature negatively modulate the growth and feed utilization; physiological conditions; hormone concentrations and immunity of *L. calbasu* and suggest that *L. calbasu* should ideally be cultured between 26 and 30 °C for optimum growth and health; making it ideal for commercial farming.

1. Introduction

An increase in the average air temperature is caused by global warming [1–4]. Fish may be directly impacted by climate change in at least two ways. First, as atmospheric CO_2 rises, acidification takes place [5], endangering fish populations [6,7]. Second, fish communities suffer from higher water temperatures linked to climate change, as evidenced by changes in species composition [1,8,9], abundance, and distribution [10,11]. The water temperature may, however, be a limiting factor for fish health because fish physiological response to variations in ambient temperature has limits [12,13].

Wildlife populations are in decline, and certain species might become extinct as a result of the effects of rising temperatures on physiological functions as growth, metabolism, and reproduction [14,15]. Fish are acclimated to water temperatures between 25 and 35 °C in the tropics [16]. Temperature can rise to a threshold where it may be detrimental to growth and physiological functions, albeit this varies from species to species [17]. Growth, which is known to be impacted by a number of biotic and abiotic factors, is one of the most crucial factors in determining the economic viability of commercial fish culture [18]. Low temperatures limit the metabolic rates of ectothermic animals, which reduces aquaculture productivity because these animals are highly sensitive to temperature changes [17]. On the other hand, it is said that higher temperatures accelerate metabolism; however, if the temperature increases too much, fish may suffer from physiological stress [19].

A useful and comparatively non-invasive method for researching fish physiology is blood. It allows for the collection of samples that can reveal information about health, biochemistry, and physiological responses to various stimuli [20]. Assessing blood chemistry provides perfect insights into the health status of fish and is a crucial tool for observing the physiological circumstances brought on by environmental influences [14,21]. The temperature of the living medium has a major effect on the haematological and biochemical components, and variations within the optimal range limit the fish's physiological health [22]. Hemato-biochemical indices can provide information on the physiological responses to environmental changes that affect homeostasis and are frequently used to evaluate the health of fish [23]. The physiological health of aquatic animals may be indicated by changes in the chemical characteristics of fish blood, which may also be an indication of temperature stress [24].

Fish hormone levels can be strongly impacted by water temperature. Fish undergo stress and increase their cortisol and adrenocorticotrophic hormone (ACTH) production when exposed to temperatures outside of their typical range. In the short term, cortisol can boost growth, immunological response, and reproductive production; however, prolonged exposure can reduce these effects [25]. Additionally, temperature has an impact on fish thyroid function, especially thyroxine (T4) and triiodothyronine (T3), which impacts fish growth, development, and survival. The degradation and conversion rates of T4 and T3 in trout and European eels increase with temperature [26]. On the other hand, an excessively low water temperature may hinder the growth of Atlantic cod by preventing the thyroid gland from doing some of its job. From early egg development to vitellogenesis and maturation, thyroid hormones have been shown to support all phases of reproduction, resulting in successful ovulation and successful spawning [27,28].

Water temperature fluctuations have a significant impact on fish immunological defense [29]. One of the main unique innate immune responses in fish is an increase in immunoglobulin M (Ig M) concentration, which is influenced by environmental stressors such pH, oxygen, salinity, and temperature [30]. Thus, the consequences of thermal stress can be assessed by measuring the Ig M concentration in fish exposed to high temperatures. It is crucial to understand that physiological changes at the molecular, cellular, and entire organism levels cause the observed effects of global warming on fish at different levels of biological organization. Ultimately, species-specific responses will determine how the ecosystem is affected by global warming [31].

The freshwater fish species *Labeo calbasu* is a member of the Cyprinidae family, which is a subfamily of the Cypriniformes order. According to Gupta et al. [32], this species of freshwater fish is primarily found in rivers, but it is also widely distributed throughout natural lakes, reservoirs, streams, ponds, beels, baors, haors, and canals. This species is significant due to its high protein content and low intramuscular bone count. It is also valued as a sport fish due to its desirable taste. Due to overfishing, habitat degradation, aquatic pollution, dam construction, and other human-caused factors that impact the fish's feeding, migrating, and spawning, the species' natural populations have drastically decreased [33]. In recent years, *L. calbasu* farming has gained attention due to decline wild population and need for species diversification in aquaculture systems. However, like many other cultured species *L. calbasu* is sensitive to water temperature, which directly affects the growth, feed conversion efficiency, and physiological performance of this species. A study by Islam et al. [34] reported that *L. calbasu* showed optimal growth, haematological parameters, immune function, and metabolic activity at 31 °C. However, temperature above 34 °C began to impair physiological and immune responses, reduce feed efficiency and caused stress, including higher glucose level and lower HB concentrations.

This study aimed to investigate the physiological responses of Kalibaus (*L. calbasu*) to acute temperature fluctuations, with a focus on growth performance, metabolic activity, haemato-biochemical responses, hormonal profiles (stress and growth-related), and immune function. The outcomes are intended to enhance understanding of the species thermal tolerance under climate change scenarios and provide a scientific basis for sustainable aquaculture practices and conservation strategies.

2. Materials and Methods

2.1. Sample Collection and Experimental Site

In this investigation, juvenile *L. calbasu* were employed, with an average body weight of 10.10 ± 0.36 g and an average total length of 10.06 ± 0.23 cm. The fish were brought to the Laboratory of Genetics and Fish Breeding at the Faculty of Fisheries, Gazipur Agricultural University (GAU), Bangladesh, from the Bangladesh Fisheries Research Institute (BFRI) in Mymensingh (Figure 1).

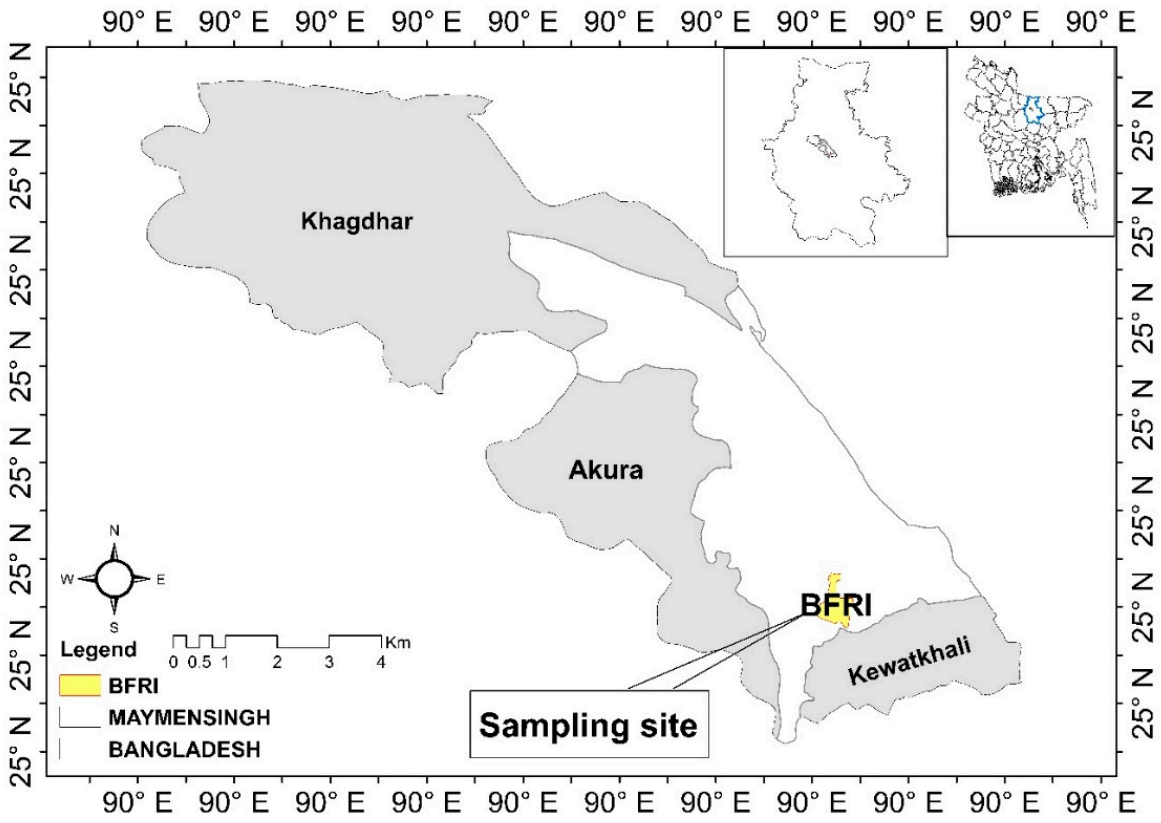

Figure 1. Map showing the sampling site.

2.2. Experimental Design

For up to 30 days, the fish were kept in equally sized stocking tanks (5000 L) at room temperature (~26 °C) and fed a commercial meal (Nourish Feeds Ltd., Bogura, Bangladesh, with 30% protein, 5% lipid, 8% crude fibre, and 12% moisture). The fish were moved to experimental tanks where the experiment was conducted for 60 days after they began feeding and excreting waste (Figure 2). With a stocking density of 15 fish per 100 L of water, tanks were randomly assigned to one of four temperature treatments (22, 26, 30, and 34 °C) with three replications. The control was set at 26 °C. A thermostat (E-JET heater 200 W, Penang, Malaysia) was used to raise the temperature changes for the experimental groups to the desired level at a rate of 2 °C day^{-1}. For self-cleaning and aeration during the study period, the tank was equipped with a filtration/aeration system (Sobo-aquarium internal filter WP-850F). The samples were kept on a 12-h light:12-h dark photoperiod for the duration of the experiment. Throughout the experiment, 20% of the water was replaced daily. Fish were sampled and data were recorded every 15 days. Fish were fed a commercial pellet diet twice a day until they were satiated at 9:00 and 16:00 (Nourish Feeds Ltd., Bogura, Bangladesh). Additionally, uneaten food and fish waste were eliminated every day.

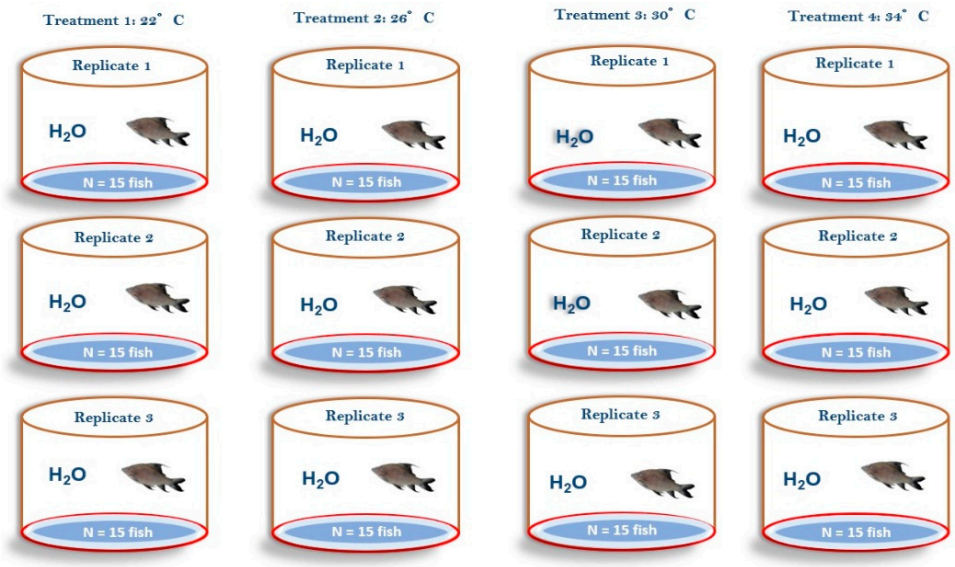

Figure 2. Experimental procedure. For 60 days, *L. calbasu* was kept under four treatments for the experiment.

2.3. Water Quality Measurements

A YSI Model 59 (Yellow Spring Instrument Company, Yellow Springs, OH, USA) was used to track the water quality parameters, such as temperature, pH, DO, and NH_3-N. Temperature and pH were taken every day at 9:00, while NH_3-N, and DO were measured every week in the morning before feeding.

2.4. Growth and Metabolic Rate Study

The fish from each experimental tank were weighed and measured separately at the start of the experiment and then every two weeks. α-methyl quinoline (TransmoreR; Nika Trading, Puchong, Malaysia) (0.22 mL L^{-1} of seawater) was used to gently anesthetize the fish for 10 to 15 min before sampling. At the end of the experiment, the final individual total length and body weight were measured to the nearest 1.0 cm and 0.01 g using a measuring board and an electronic balance (Model: KD-300KC) [35]. Growth and metabolic indices viz. total length (cm), body weight (g), total length gain (cm), body weight gain (g), food conversion ratio, food conversion efficiency, condition factor, and survival rates were measured using the following formulas:

$$\text{Body weight gain (BWG, g)} = (W_2 - W_1) \times n \quad (1)$$

$$\text{Food conversion ratio (FCR)} = F/(W_2 - W_1) \quad (2)$$

$$\text{Food conversion efficiency (FCE)} = (\text{gain in fish mass} \times \text{g food consumed}^{-1}) \quad (3)$$

$$\text{Condition factor (CF)} = W2 \times 100\ L2^{-3} \quad (4)$$

$$\text{Survival rate \%} = [\text{Final number of fish/initial number of fish}] \times 100 \quad (5)$$

Here, L1 & L2 and W1 & W2 represent the starting (0 d) and final (60 d) average individual length and weights, respectively, per treatment, *n* represents the final number of fish, and F represents the total feed consumption during the experimental period.

2.5. Haematological Indices Study

The fish were anesthetized with α-methyl quinoline (Transmore®; Nika Trading, Puchong, Malaysia) after being starved for 24 h after the experiment. Using a sterile plastic syringe (2.5 mL), blood samples were drawn from the caudal vein of each experimen-

tal group's fish. The samples were then stored in two separate tubes: one (Miniplast 3.0 mL; LP Italiana Spa, Milan, Italy) that contained ethylenediamine tetraacetic acid (EDTA, 1.26 mg/0.6 mL) as an anticoagulant agent and another without EDTA for serum collection. Using an automated haematology analyzer (HeCo Vet C; SEAC, Florence, Italy), the haematological profile—which includes red blood cell count (RBC), white blood cell count (WBC), haemoglobin (HB), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), and platelets (PLT)—was determined using the blood samples collected in EDTA tubes [36].

2.6. Serum Biochemical Parameters Study

The blood was drawn and allowed to coagulate in sterile eppendorf tubes. For ten minutes, the clotted blood was centrifuged at 12,000 rpm. For further examination, a clear fluid serum-was pipetted into a 1.5 mL sterile eppendorf tube [37]. Assay kits (Biosino Bio-technology and Science Inc., Beijin, China) were used to measure total protein (TP, g d L^{-1}), albumin (ALB, g dL^{-1}), globulin (GLB, g dL^{-1}), alkaline/globulin ratio (A/G ratio), HCO_3 (TCO_3), alkaline phosphatase (AP, IU L^{-1}), Ast/SGOT (U L^{-1}), and alanine transaminase ALT/SGPT (U L^{-1}) [37]. A diagnostic reagent kit provided by Monozyme, In-vitro Diagnostics, Hyderabad, India, was used to assess the levels of sodium (Na^+, mmol L^{-1}), potassium (K^+, mmol L^{-1}), and chloride (Cl^-, mmol L^{-1}).

2.7. Hormonal Concentration Study

Fish serum was extracted from three replicated fish per treatment for a hormonal analysis, similar to that in the biochemical parameter investigation. The enzyme-linked immunosorbent assay (ELISA), as described by [38], was used to detect growth hormone (GH), cortisol, adrenocorticotropic hormone (ACTH), and thyroid hormones (T3, T4) using test kits that were acquired from Uscnlife (Wuhan, China).

2.8. Immune Responses Study

Enzyme-linked immunosorbent assay (ELISA) quantification kit (MyBioSource Inc., San Diego, CA, USA) was used to quantify the concentration of plasma immunoglobulin M (IgM). Plasma samples and IgM standards were examined manually. Following all procedures, the optical density was measured in 15 min at 450 nm.

2.9. Statistical Analysis

Since there were no discernible differences between any of the replicate averages ($p > 0.05$), the data for each replicate were averaged [39]. Before statistical analysis, all data were examined for homogeneity of variances using Bartlett's test [40] and for normality of distribution using the Kolmogornov-Smirnov test [41]. This was performed using a Student-Newman-Keuls multiple comparison test and one-way analysis of variance (ANOVA). A pairwise post hoc Tukey test was performed to identify the precise groups that differed when the ANOVA revealed significant differences [41]. Unless otherwise indicated, a significance level (α) of 0.05 was applied. All information is displayed as mean values ± SE. Minitab™ software version 17 and Origin™ software version 9.0 were used for all statistical analyses.

3. Results

3.1. Water Quality Measurements

Table 1 presents the mean values along with the standard error (SE) of the water quality parameters measured in the experimental tanks throughout the study period. All

metrics exhibited no significant differences ($p > 0.05$) and remained adequately stable at the standard treatment temperatures of 22, 26, 30, and 34 °C.

Table 1. Physico-chemical parameters monitored during the experiment.

Water Quality Parameter	Temperatures (°C)			
	22	26	30	34
pH	7.5 ± 0.2 [a]	7.5 ± 0.4 [a]	7.7 ± 0.3 [a]	7.7 ± 0.2 [a]
DO (mg L^{-1})	5.4 ± 0.20 [a]	5.5 ± 0.14 [a]	5.6 ± 0.66 [a]	5.3 ± 0.19 [a]
NH_3-N (mg L^{-1})	0.22 ± 0.03 [a]	0.24 ± 0.04 [a]	0.19 ± 0.02 [a]	0.15 ± 0.01 [a]

Means ± SE are used for values. Within the same row, mean values that are followed by a common superscript letter do not differ significantly ($p < 0.05$).

3.2. Effect of Temperature on Growth and Metabolism

The mean initial weight and length values for the four temperatures (22, 26, 30, 34) were similar (Table 2). This experiment showed that the water temperature substantially affected the fish's FBW, TLG, BWG, FCE, and FCR. Fish maintained at 30 °C exhibit the highest FTL, FBW, and BWG values (11.26 ± 0.12, 12.78 ± 0.12, and 3.07 ± 0.70, respectively). Fish grown at 22 and 34 °C had significantly higher FCR values (1.91 ± 0.05 and 1.83 ± 0.07) than fish raised at 26 and 30 °C (1.62 ± 0.04 and 1.51 ± 0.03; $p < 0.05$, Table 2). The FCR values gradually decreased as the temperature was raised from 22 to 30 °C, but they significantly increased when the temperature was raised to 34 °C. Overall, the best FCR performance was shown by the group that was raised at 30 °C. The optimal temperature for FCE, on the other hand, was estimated to be 0.66 ± 0.00 and 30 °C.

Table 2. Growth performances of *L. calbasu* (Mean ± SE).

Variables	Temperature (°C)			
	22	26	30	34
ITL	10.38 ± 0.18 [a]	9.75 ± 0.16 [a]	9.97 ± 0.11 [a]	10.15 ± 0.11 [a]
FTL	10.94 ± 0.11 [a]	10.74 ± 0.14 [a]	11.26 ± 0.12 [a]	10.87 ± 0.10 [b]
IBW	10.6 ± 0.28 [a]	9.8 ± 0.10 [b]	9.71 ± 0.10389 [b]	10.28 ± 0.06 [a]
FBW	12.29 ± 0.28 [b]	12.37 ± 0.25 [b]	12.78 ± 0.12 [a]	12.19 ± 0.11 [b]
TLG	0.56 ± 0.11 [b]	0.99 ± 0.17 [a]	1.29 ± 0.15 [a]	0.72 ± 0.09 [b]
BWG	1.69 ± 0.36 [b]	2.57 ± 0.21 [ab]	3.07 ± 0.20 [a]	1.91 ± 0.51 [b]
FCR	1.91 ± 0.05 [c]	1.62 ± 0.06 [a]	1.51 ± 0.13 [a]	1.83 ± 0.07 [b]
FCE	0.52 ± 0.05 [b]	0.62 ± 0.02 [a]	0.66 ± 0.03 [a]	0.55 ± 0.10 [ab]
CF	0.93 ± 0.08 [a]	1.02 ± 0.20 [a]	0.92 ± 0.19 [a]	0.96 ± 0.15 [a]
Sur	100	100	100	100

Means ± SE are used for values. Within the same row, mean values that are followed by a common superscript letter do not differ significantly ($p < 0.05$).

3.3. Effect of Temperature on Haematological Parameters

The present study revealed that different temperatures have a strong influence on fish haematology. At 30 °C some haematological parameters were higher ($p < 0.05$) such as RBC (1.42 ± 0.20), Haemoglobin (8.47 ± 0.81), HCT (42.67 ± 1.70) than other three temperature (Table 3). WBC values were highest at 22 and 34 °C (1.73 ± 0.07; 1.58 ± 0.04, respectively) whereas HCT level lowest at the same temperature (17.67 ± 2.87; 24.00 ± 1.63, respectively). MCV found highest at 26 and 30 °C (332.06 ± 29.97; 307.69 ± 46.76, respectively). For MCV, no notable difference observed between 22 and 34 °C ($p > 0.05$). On the other hand, MCH, MCHC, PLT were higher ($p < 0.05$) at 22 °C (3.28 ± 0.57, 32.80 ± 5.65, 4.31 ± 0.42, respectively).

Table 3. Haematological parameter of the blood of *L. calbasu* (Mean ± SE).

Blood Parameter	Temperature (°C)			
	22	26	30	34
RBC ($\times 10^6$ mm^{-3})	0.64 ± 0.05 ^{d}	1.05 ± 0.14 ^{b}	1.42 ± 0.20 ^{a}	0.85 ± 0.04 ^{c}
WBC	1.73 ± 0.07 ^{a}	1.12 ± 0.03 ^{c}	0.86 ± 0.03 ^{d}	1.58 ± 0.04 ^{b}
Haemoglobin (g dL^{-1})	5.63 ± 0.12 ^{c}	6.87 ± 0.49 ^{b}	8.47 ± 0.81 ^{a}	6.23 ± 0.26 ^{b}
HCT (%)	17.67 ± 2.87 ^{d}	34.33 ± 1.70 ^{b}	42.67 ± 1.70 ^{a}	24.00 ± 1.63 ^{c}
MCV	277.87 ± 43.30 ^{b}	332.06 ± 29.97 ^{a}	307.69 ± 46.76 ^{ab}	282.21 ± 26.99 ^{b}
MCH (pg)	3.28 ± 0.57 ^{a}	2.00 ± 0.06 ^{c}	1.99 ± 0.25 ^{c}	2.60 ± 0.12 ^{b}
MCHC (g dL^{-1})	32.80 ± 5.65 ^{a}	19.98 ± 0.61 ^{b}	19.92 ± 2.49 ^{b}	26.04 ± 1.21 ^{a}
PLT ($\times 10^5$)	4.31 ± 0.42 ^{a}	2.44 ± 0.16 ^{b}	3.96 ± 0.46 ^{a}	1.08 ± 0.02 ^{c}

Means ± SE are used for values. Within the same row, mean values that are followed by a common superscript letter do not differ significantly ($p < 0.05$).

Figure 3 shows the association between haematological parameters and temperature using robust hierarchical co-clustering. The color intensity reflects the scaled values of haematological parameters across samples. White areas show lower values, whereas black areas show greater values. Haematological parameters such as RBC, HB, HCT had strong association, so they were clustered closely, possibly indicating they respond similarly to 30 °C temperature. These parameters were also closely clustered at 26 °C temperature but the intensity was lower compared to 30 °C. On the other hand, MCHC, MCH and WBC showed close clustered and strong association at 20 °C and 34 °C temperatures. The case of MCV and PLT showed cluster in response to 26 °C and 30 °C temperature.

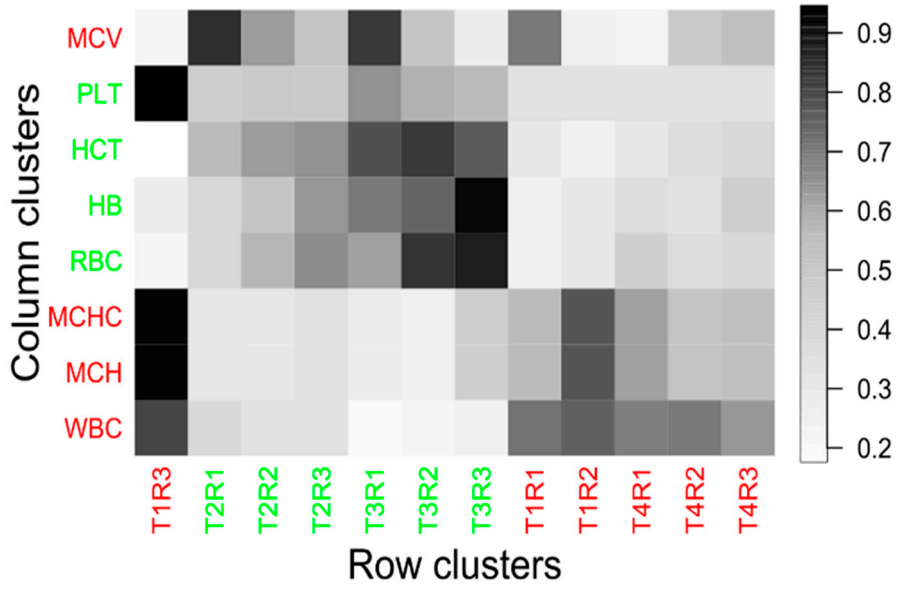

Figure 3. Haematological parameters and temperature relationship are indicated by robust hierarchical co-clustering (method = ward. D2 and distance = Manhattan). The stress tolerance index (STI) values derived from the parameters under study were grouped and normalized. The treatment (temperature) level produced four-row clusters (row clusters 1, 2, 3, and 4), whereas the parameter level produced three-column clusters (column clusters 1, 2, and 3). The cluster numbers were established using the machine language of the gap statistic. The intensity of the modified STI values of the parameters is expressed by various greyscale tones. Variables highlighted in red and green denote groups of physiological indicators responding distinctly among treatments, illustrating treatment-specific clustering patterns.

Figure 4 displays the collected principal component analysis (PCA) biplot of the relationship among haematological parameters and their association with four treatments. The first (PC1) and second (PC2) components accounted for 66.9% and 21.1% of the total variance (88%). Blood parameters such as HB, RBC, HCT are strongly and positively correlated, indicating similar direction along PC1. MCV had primarily along PC2 while WBC and MCHC appeared negatively correlated with RBC related parameters based on their opposite orientation. RBC and HB were positively correlated for treatment T3 because they formed a small angle and strongly influenced on first principal component (PC1). Since WBC and PLT met each other at 90° angle, they were not likely to be correlated.

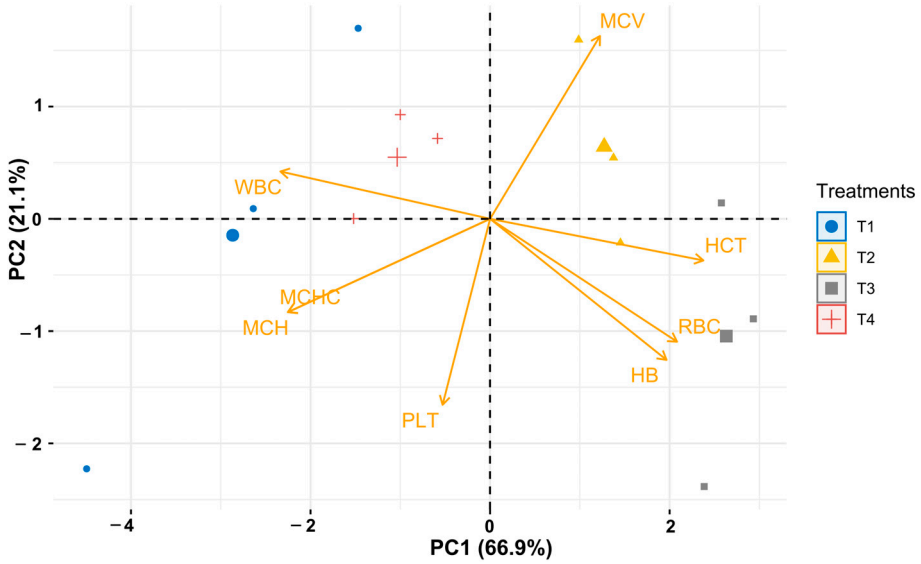

Figure 4. The association between treatments and observed haematological parameters is specified by a PCA biplot. The direction of the rising values for each factor is indicated by the yellow arrows. Depending on how divergent they are from one another, parameters are distributed in various ordinates. The temperature and haematological parameters of a vector in this biplot show the proportion of the parameters to the principal components and the quality of representation, respectively. Whether the qualities interacted favorably or unfavorably is indicated by the angles between the vectors created from the biplots' midway point.

3.4. Effect of Temperature on Biochemical Responses

At 30 °C some parameters such as albumin (1.84 ± 0.05 g dL^{-1}), globulin (1.64 ± 0.06 g dL^{-1}), HCO_3 (30.93 ± 0.62), Na^+ (115.60 ± 3.72 mmol L^{-1}), alkaline phosphate (93.33 ± 9.39 IU L^{-1}), Ast/SGOT (21.00 ± 4.55 IU L^{-1}) were higher ($p < 0.05$) than other three temperatures (Table 4). Total Protein, A/G ratio, K^+, ALT/ SGPT were higher ($p < 0.05$) at 34 °C (2.98 ± 0.03 g dL^{-1}, 1.50 ± 0.11, 2.15 ± 0.04 mmol L^{-1}, 19.33 ± 1.25 UL^{-1}, respectively) and between 22 and 34 °C, there is no discernible change ($p > 0.05$). (Table 4).

Table 4. Biochemical parameter of the blood of *L. calbasu* (Mean ± SE).

Parameters	Temperature (°C)			
	22	26	30	34
Total Protein (g d L^{-1})	2.95 ± 0.05 ^{a}	2.78 ± 0.04 ^{b}	2.96 ± 0.03 ^{a}	2.98 ± 0.03 ^{a}
Albumin (g d L^{-1})	1.15 ± 0.05 ^{d}	1.67 ± 0.09 ^{b}	1.84 ± 0.05 ^{a}	1.43 ± 0.10 ^{c}
Globulin (g d L^{-1})	0.88 ± 0.04 ^{c}	1.45 ± 0.05 ^{b}	1.64 ± 0.06 ^{a}	0.95 ± 0.05 ^{c}
A/G ratio	1.32 ± 0.12 ^{a}	1.16 ± 0.09 ^{b}	1.12 ± 0.02 ^{b}	1.50 ± 0.11 ^{a}
HCO_3 (TCO_3)	28.33 ± 0.92 ^{b}	30.77 ± 0.48 ^{a}	30.93 ± 0.62 ^{a}	28.83 ± 1.27 ^{b}

Table 4. *Cont.*

Parameters	Temperature (°C)			
	22	26	30	34
Alkaline Phosphate (AP, IU L^{-1})	69.00 ± 6.48 ^{b}	81.33 ± 3.30 ^{a}	93.33 ± 9.39 ^{a}	80.00 ± 8.83 ^{a}
Ast/SGOT (U L^{-1})	16.00 ± 1.63 ^{b}	17.67 ± 5.25 ^{ab}	21.00 ± 4.55 ^{a}	12.33 ± 2.05 ^{c}
ALT/SGPT (U L^{-1})	16.07 ± 0.87 ^{b}	17.67 ± 3.09 ^{ab}	18.60 ± 0.54 ^{a}	19.33 ± 1.25 ^{a}
Na^{+} (mmol L^{-1})	84.57 ± 5.48 ^{b}	89.48 ± 7.17 ^{ab}	115.60 ± 3.72 ^{a}	96.13 ± 4.97 ^{b}
K^{+} (mmol L^{-1})	1.97 ± 0.04 ^{b}	1.95 ± 0.07 ^{c}	2.06 ± 0.06 ^{a}	2.15 ± 0.04 ^{a}
Cl^{-} (mmol L^{-1})	59.13 ± 2.41 ^{c}	79.43 ± 5.53 ^{a}	78.27 ± 1.77 ^{a}	67.10 ± 2.54 ^{b}

Means ± SE are used for values. Within the same row, mean values that are followed by a common superscript letter do not differ significantly ($p < 0.05$).

Figure 5 Shows the association between biochemical parameters and temperature using robust hierarchical co-clustering. Biochemical parameters such as albumin, globulin, HCO_3, Na, Cl, AP, IgM, T3, T4, E2, Testosterone, ACTH, had strong association, so they were clustered closely, possibly indicating they respond similarly to 30 °C temperature. On the other hand, cortisol, K, A/G ratio and TP had strong association, and clustered closely, reflecting a similar response at 34 °C temperature. In the case of, ALT and AST remained closely and showed strong association with higher STI value at 34 °C and 30 °C temperature.

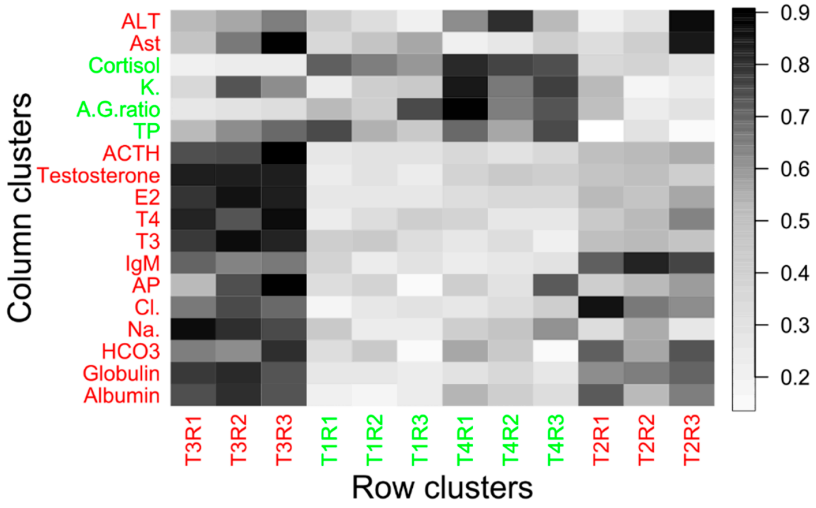

Figure 5. The relationship between biochemical characteristics and temperature is demonstrated through strong hierarchical co-clustering (method = ward. D2, distance = Manhattan). The stress tolerance index (STI) values derived from the studied parameters were grouped and normalized. The temperature treatments created four row clusters (row clusters 1, 2, 3, and 4), while the parameters formed three column clusters (column clusters 1, 2, and 3). The cluster numbers were determined using the machine learning technique of the gap statistic. The varying intensities of the modified STI values for the parameters are represented by different greyscale shades. Variables highlighted in red and green denote groups of physiological indicators responding distinctly among treatments, illustrating treatment-specific clustering patterns.

Figure 6 displays the collected principal components as a PCA biplot of the variables. Where the first (PC1) and second (PC2) components accounted for meaningful amounts of the total variance (76.5%). PC1 expressed 61% of the total variance and exhibited positive loadings for albumin, globulin, HCO_3, Na, Cl, AP, IgM, T3, T4, E2, Testosterone, ACTH. On the other hand, negative loadings were noted for cortisol, A.G. ratio, TP and K. Biochemical parameters such as TP, K, AP loaded strongly and positively in PC2 that exhibited 15.5%

of the total variance. In this figure, hormones including ACTH, testosterone, E2, T3, T4 along with NA, Cl, HCO_3 and IgM had strong correlation, contributing significantly to the separation of T2 and T3 treatments. Meanwhile, T1 showed a strong negative association with these parameters. In contrast, T4 separates PC2 with high concentration of cortisol, A/G ratio.

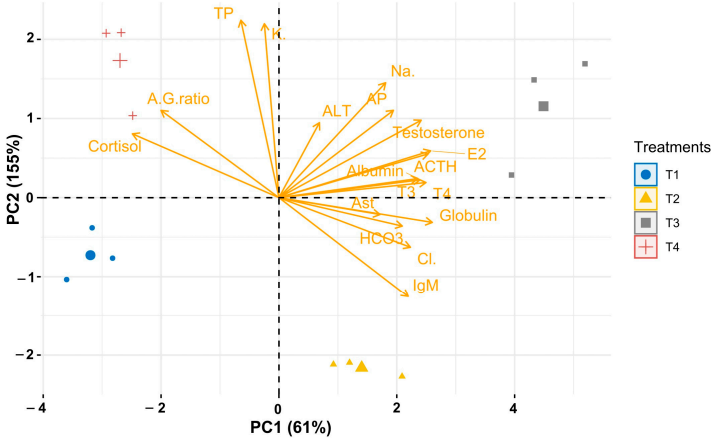

Figure 6. The association between treatments and observed biochemical parameters is specified by a PCA biplot. The direction of the rising values for each factor is indicated by the yellow arrows. Depending on how divergent they are from one another, parameters are distributed in various ordinates. In this biplot, a vector's temperature and biochemical properties show how well it is represented and how much it contributes to the principal components. Whether the qualities interacted favorably or unfavorably is indicated by the angles between the vectors created from the biplots' midway point.

3.5. Effect of Temperature on Hormonal Activity

3.5.1. Impacts of Water Temperature Fluctuations on Growth Hormone Levels

The growth hormone (GH) was lower at 22 °C. As the temperature increased, GH levels also rose significantly ($p < 0.05$) and the highest level was found at 30 °C, but after 30 °C, it can fall and lower growth was found at 34 °C (Figure 7). The polynomial cubic relationship between GH and temperature (T) could be described as $GH = 0.7623 - 0.5566T + 0.59895T^2 - 0.10505T^3$ and revealed that the relation between water temperature and GH is quite significant ($r^2 = 0.998$, $p < 0.05$).

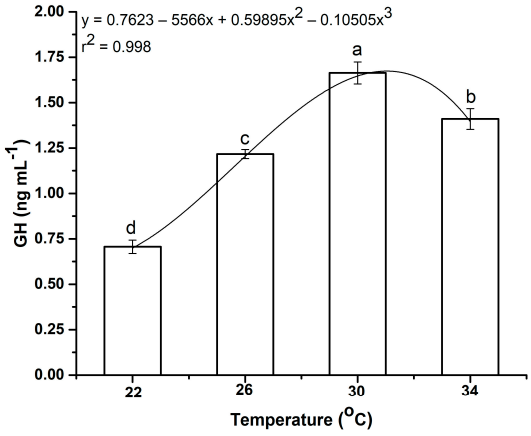

Figure 7. Relationship between Growth Hormone (GH, ng mL^{-1}) and temperature (°C). Values are presented as mean ± SE (n = 15). Patterns with various letters differ significantly ($p < 0.05$). The line defines the polynomial cubic model fitted to the mean data to show the general trends in growth hormone with temperatures.

3.5.2. The Effects of Water Temperature Change on Serum Cortisol and Adrenocorticotropic Hormone (ACTH) Levels

The current study used variations in serum cortisol levels to track how changes in water temperature affected *L. calbasu*'s stress response. The effects on fish serum cortisol concentrations after water temperature increases from 22 to 26, 30, and 34 °C, respectively, are depicted in Figure 8a. Results illustrate that at lower temperatures (22 °C) serum cortisol level was highest (1.62 ± 0.06 µg dL^{-1}). By increasing temperature, the cortisol concentrations decreased to 30 °C (1.07 ± 0.03 µg dL^{-1}). But at 34 °C suddenly serum cortisol was found at its highest level (1.81 ± 0.06 µg dL^{-1}). Nevertheless, there is no substantial variation between 22 and 34 °C.

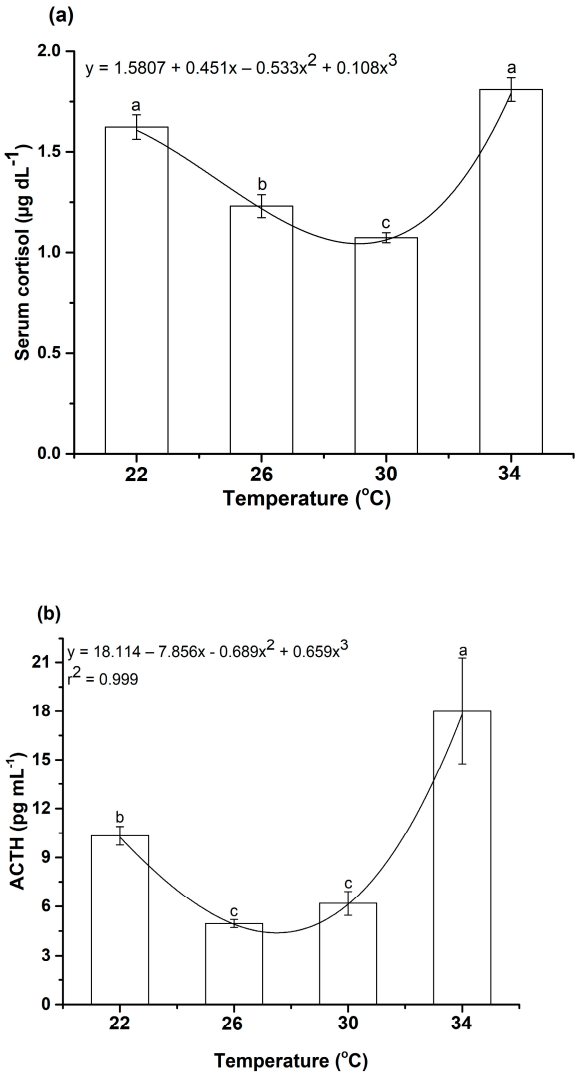

Figure 8. Relationship between (**a**) cortisol (µg dL^{-1}) and (**b**) adrenocorticotropic Hormone (ACTH) with temperature (°C) acclimated to four distinct temperatures (22, 26, 30 and 34 °C). Significant differences exist between patterns with different letters ($p < 0.05$). The line illustrates the general patterns in cortisol and ACTH with temperature by fitting a polynomial cubic model to the mean data.

The decrease and/or increase in temperature induces a transient increase in ACTH level (Figure 8b). When the temperature increased from the control temperature (26 °C) the ACTH level increased and achieved its maximum temperature of 34 °C (18.01 ± 3.26 (pg mL^{-1}). Again, the ACTH level also increased with decreasing temperature to 22 °C (10.33 ± 0.57 pg mL^{-1}). The plasma ACTH value at 34 °C was significantly increased from

the other treated fishes ($p < 0.05$). Nevertheless, there is no discernible difference between fish treated at 26 and 30 °C ($p > 0.05$). ACTH levels peaked in fish reared at 34 °C, followed by 22 °C, 30 °C, and 26 °C (Figure 8b).

3.5.3. Effects of Water Temperature Changes on Thyroid Hormones

The free T3 and T4 levels displayed variations with temperature fluctuations (Figure 9). Initially, the T3 and T4 were found lower at 22 °C (0.84 ± 0.053 ng mL^{-1} and 1.14 ± 0.10 µg dL^{-1} respectively). With increasing temperature, both T3 and T4 increase exponentially up to 30 °C. After that, the thyroid hormones secretions started to reduce and reached the lowest level (0.67 ± 0.09 ng mL^{-1} and 1.1 ± 0.09 µg dL^{-1} respectively). The highest level of T3 was found at 30 °C (1.44 ± 0.07 ng mL^{-1}) and significantly differed from the other treatments ($p < 0.05$). However, no noteworthy difference was revealed between 22 °C and 34 °C ($p > 0.05$). Similar trends also emerged in the instance of T4.

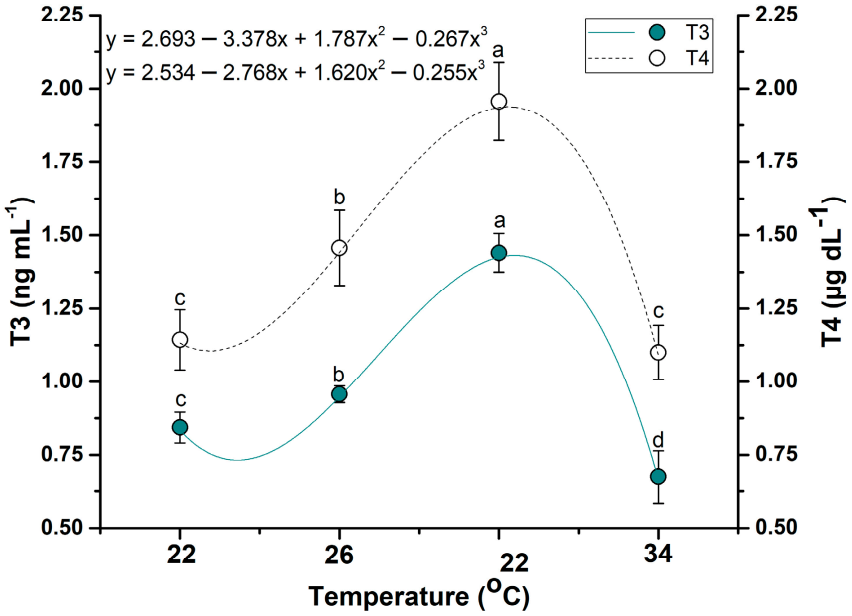

Figure 9. T3, T4, and temperature adapted to four distinct temperatures (22, 26, 30, and 34 °C) are related. Values are shown as mean ± SE (n = 15), and trends have significant variation ($p < 0.05$) with different letters. Filled circles represent data for T3 and white circles represent data for T4. The solid and broken lines illustrate the general patterns in T3 and T4 with temperature by fitting a polynomial cubic model to the mean data.

3.6. Effect of Temperature on Immunology

On the immune level, serum fish immunoglobulin M (IgM) levels were decreased in fish grown at lower (22 °C) and higher (34 °C) Temperature (Figure 10). It is notable that the highest fish immunoglobulin M (IgM) shown in fish raised at 30 °C (1.107 ± 0.079 ug mL^{-1}) while the lowest level was recorded at 34 °C (0.45 ± 0.025 ug mL^{-1}). There is no notable difference between the fishes reared at 26 and 30 °C. In the same way, fish housed at 22 and 34 °C did not differ significantly ($p > 0.05$). However, there were notable differences between the results at 26 and 30 °C and those at 22 and 34 °C.

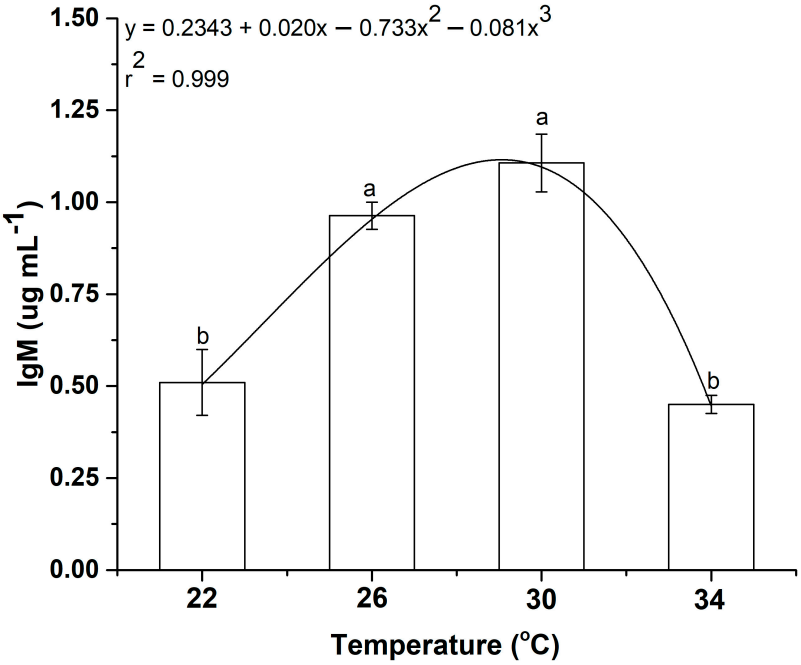

Figure 10. Relationship between IgM and temperature. Mean ± SE (n = 15) is used to express values. Significant differences exist between trends with different letters ($p < 0.05$). The line defines the polynomial cubic model fitted to the mean data to show the general trends in immunoglobulin M with temperatures.

4. Discussion

Stress in fish is broadly defined as the physiological response to threatening or adverse conditions, similar to other vertebrates [42]. It occurs when environmental factors deviate from the optimal range, impairing normal homeostatic regulation. Among these factors, water temperature plays a particularly significant role in influencing various physiological functions. Each fish species has a specific temperature range within which it operates efficiently, and deviations from this range can induce stress, interfering with normal behavior and biological processes [43]. In this experiment, several parameters measured (growth, metabolism, blood chemistry, growth hormone, cortisol, ACTH, T3, T4 levels, and immunoglobulin) were found to be rather stable at the control temperature (26 °C), suggesting that *L. calbasu* adapted well to this temperature. As a result, any alteration from this control temperature will lead to temperature-specific changes in how various features manifest.

Temperature influenced growth in this research, with the highest growth rate recorded at 30 °C. Throughout the experiment, temperatures ranged from 26 °C to 30 °C, during which the fish showed no adverse effects such as irreversible damage, loss of equilibrium, respiratory arrest, or reduced food intake. Due to the effect on feed intake, food digestion time, and enzyme activity, both FC and FCR decrease and increase when the temperature deviates from the ideal range [44]. These results may help to explain why the experiment's FC and FCR values at 30 °C were satisfactory. The Malabar blood snapper showed good FCR values at 26 and 30 °C, which was in line with this result [44,45]. Katersky and Carter [46] showed that SGR peaked in *Lates calcarifer* between 33 and 36 °C, and that there was no appreciable change when the temperature increased from 27 to 36 °C. They also found that growth efficiency decreased as the temperature increased over 36 °C.

Finding information about the general health of the fish species requires knowledge of the haematological indicators. Stress alters the haematological parameters of fish, with downstream impacts on numerous physiological processes [14]. The results of the study

showed that both high and low temperatures had a notable impact on the blood parameters of *L. calbasu*. The current results showed that at 30 °C, there was an increase in RBC, hemoglobin, HCT, and MCV. Fish have been shown to have a propensity to produce more hemoglobin and red blood cells as the temperature rises [47]. According to Witeska et al. [48], the WBC level is a known measure of fish health and is crucial for boosting innate or nonspecific immunity. The current study's WBC content increased significantly at both the higher and lower temperature regimes (22 and 34 °C), which may encourage the production of antibodies in response to a stressful environment. The values observed in our current experiment are consistent with those found in *Lutjanus malabaricus* by Mazumder et al. [35] and *Cyprinus carpio* by [49]. Abnormal increases in WBC count at both lower and higher temperatures may be a sign that animal physiology is under thermal stress [50].

Serum biochemical characteristics are thought to be a reliable diagnostic method for assessing fish health and nutrition [51,52]. The fish in this study had reduced levels of total protein, albumin, and globulin after being exposed to both hot (34 °C) and cold water (22 °C). Yilmaz et al. [53] observed similar findings in *O. mossambicus*, *O. niloticus*, and red hybrid *O. niloticus*. According to the current study, *L calbasu*, which is most impacted by cold and hot water stress, has the largest levels of albumin and globulin, which suggests that the fish's survival mechanisms are more active. Variations in the water temperature in this study caused *L. calbasu*'s hydromineral status to respond in a number of ways, as evidenced by variations in plasma osmolality and Na^+, K^+, and Cl^- values. Temperature variations had an impact on plasma osmolality, which rose as the temperature rose. The decreased temperature, however, had the most noticeable effect on the plasma ion levels (22 °C). Similarly, with Gilthead seabream (*Sparus aurata*) cultivated in earthen ponds, Vargas-Chacoff et al. [54,55] observed a positive correlation between temperature fluctuation and osmoregulatory performance. AST and ALT are important enzymes in assessing liver injury. When liver and heart cells are injured or their permeability increases, AST and ALT are released into the bloodstream, which raises blood transaminase activity [56]. In this research, the AST levels decreased with cold and hot water stress though, ALT level increased with increasing temperature. According to Zhou et al. [56], *O. niloticus*'s AST and ALT levels rose following a 6-h, 8 °C cold water stress. High temperatures decrease ionic regulation through the determination of transporter proteins in gills. Furthermore, differential expression of genes in processes related to oxidative stress, protein degradation, metabolism, cell trafficking, and ionic transport have been reported in the transcriptome of gill and kidney tissue in Chinook salmon (*Oncorhynchus tshawytscha*) and rainbow trout (*Oncorhynchus mykiss*) exposed to different temperatures [57,58].

Water temperature also affects the hormonal regulation of growth and stress responses in fish. Growth hormone (GH) levels, which are key regulators of fish growth, were found to be lower at 22 °C and higher at 30 °C, aligning with the optimal growth conditions observed in this study. However, at temperatures above 30 °C, the GH levels decreased, resulting in reduced growth. This pattern mirrors the findings of Mingarro et al. [59] and Figueroa et al. [60], who observed similar trends in other fish species, such as gilthead sea bream and carp.

The levels of the vertebrate stress hormones cortisol and ACTH are essential biochemical markers for assessing how stressed-out fish are [61,62]. The present findings showed that at lower temperatures (22 °C) cortisol and ACTH levels were higher. By increasing temperature, the cortisol concentrations decreased to 30 °C. But again at 34 °C, cortisol and ACTH levels increased dramatically. The behavioural alterations seen in this study are comparable to those seen in other species, including silver barbs (*Barbonymus gonionotus*) and Atlantic salmon (*Salmo salar*) [33,63]. ACTH is the primary modulator of cortisol secretion [64]. To counteract or lessen the negative effects of stressors,

fish frequently use their liver's protein stores for gluconeogenesis to produce more cortisol and ACTH under stressful conditions [48,62,65,66]. The detrimental effects of temperature fluctuations are demonstrated by the significantly higher cortisol and ACTH levels of *L. calbasu* under various temperature treatments during the course of the experiment. Higher cortisol and ACTH levels in the treatment groups until the end of the experiment further support the idea that *L. calbasu* in different temperature treatment groups were under continuous stress. According to Faught and Schaaf [67], stress impacts metabolic pathways and immune responses in fish by activating the Hypothalamus-pituitary interrenal (HPI) axis, which releases stress hormones like cortisol. These hormones bind to glucocorticoid and mineralocorticoid receptors on immune cells, modulating inflammation by regulating pro-inflammatory and anti-inflammatory gene expression. This interaction can suppress or enhance immune function, highlighting the complex role of stress in immune regulation.

A variety of external events and the physiological condition of fish may influence the relationship between thyroid hormone concentrations and water temperature [68]. Thyroid hormones, including T3 and T4, also play a key role in regulating metabolic processes and thermal adaptation. Our findings demonstrated that the concentrations of these hormones were lower in both cold (22 °C) and hot (34 °C) water, indicating that extreme temperatures impair the fish's ability to acclimate metabolically. Similar results have been reported for rainbow trout [68] and carp [69], further emphasizing the role of thyroid hormones in thermal acclimation.

Immune function is a critical aspect of fish health, particularly in aquaculture settings where diseases can spread rapidly in dense farming environments. Immunoglobulin M (IgM), a key component of the immune system, was found to be highest in fish maintained at 30 °C, suggesting that this temperature supports optimal immune function. In contrast, both lower (22 °C) and higher (34 °C) temperatures resulted in decreased IgM levels, indicating compromised immune defense. These findings are consistent with research by Dominguez et al. [70], who reported similar trends in Nile tilapia, emphasizing the importance of maintaining a stable and optimal temperature for immune function in farmed fish.

5. Conclusions

In summary, the investigation of the extreme ambient temperature-mediated alterations in Kalibaus (*Labeo calbasu*) offers important new information about the physiological and biochemical responses of this species to heat stress. The results show notable changes in metabolic rates, hemato-biochemical indicators, and stress-induced reactions, underscoring the species' susceptibility to temperature fluctuations. Notably, the hormonal responses show a disturbed homeostasis, which may affect overall health and stress, especially when it comes to growth and stress hormones. Furthermore, the immunological responses reveal a weakened defensive system, highlighting the negative consequences of temperature-induced stress on *L. calbasu*. Based on the aforementioned results, it can be concluded that a water temperature of 30 °C is most favorable for the optimal growth of *L. calbasu* and can be recommended for achieving the best growth performance in aquaculture systems. However, maintaining a consistent water temperature remains a significant challenge in practical aquaculture settings. To address this, a collaborative effort between industry stakeholders and aquaculture experts is necessary to develop cost-effective and efficient solutions for stabilizing water temperature in aquafarms, ultimately enhancing fish growth and farm productivity. The maximal temperature acclimatization and long-term adaptive responses to this species' fitness in a changing climate situation, however, require more research. Furthermore, the results of this study support the need for more defensive

signalling pathway research to help develop new cultivars that are better suited to drier climates of the future.

Author Contributions: S.K.M., M.L.R. and S.K.D. conceived and designed the experiments. M.S.M., S.R.D., M.N.H. and M.A.S. performed the statistical analysis and prepared the manuscript, the table and the figures. M.S.M., T.R. and M.N.I. conducted the experiment. M.N.I., T.R., S.R.D. and M.L.R. collected the samples. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: All fish experiments in the present study were approved by the Research Management Wing of Gazipur Agricultural University, Bangladesh (Approval Code: GAU/RMW/2022/134(KA)6068, Approval date: 22 April 2025).

Informed Consent Statement: Not applicable.

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Conflicts of Interest: The authors declare no conflicts of interest.

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