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Effects of water temperature and diet on blood parameters and stress levels in hybrid grouper (*Epinephelus fuscoguttatus* ♀ × *E. lanceolatus* ♂) juveniles

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ABSTRACT

Hybrid grouper (*Epinephelus fuscoguttatus* [TG] × *E. lanceolatus* [GG]) juveniles were evaluated in terms of their blood hematology and biochemical levels after 60 days of exposure to different temperatures (22 °C, 26 °C, 30 °C, and 34 °C) in triplicate (20 fish/tank; body weight 200 ± 15 g; length 22.5 ± 1 cm). The fish were fed daily with commercial pellets (42% protein, 9% ash, and 11% lipid) or shrimp (42% protein, 9% ash, and 11% lipid). Results showed that the blood hematologic parameters of the fish fed with shrimp at 26 °C were significantly better than those at other temperatures. The lowest stress level measured in terms of glucose (26 ± 10 mg dL^{-1}) and cortisol concentrations (0.5 ± 0.1 ng mL^{-1}) after 60 days of the experiment were observed in the fish fed with shrimp at 26 °C compared with those fed with other diets and at other temperatures. Overall, the TGGG hybrid showed the highest red blood cell and total protein counts in fish fed with a shrimp diet at 26 °C, which were healthful and less stressful conditions. Thus, the health of TGGG hybrid fed with shrimp was better than that of fish fed with other diets and at other temperatures. These findings would be useful for further studies on the management of the TGGG hybrid to increase production.

1. Introduction

Examining blood parameters has been considered a valuable approach for analyzing the health status of fish and enhancing our understanding of the relationship of blood characteristics with the habitat and adaptability of a species to an environment (Satheeshkumar et al., 2012; Fazio et al., 2012; Acar and Türker, 2018; Parrino et al., 2018; Noor et al., 2019). The physiological and biochemical characteristics of fish blood are easily modified by temperature, ecological habitat, food selection, and mode of life; therefore, these parameters are considered useful factors for fish health analysis (Maceda-Veiga et al., 2010; Ferguson et al., 2010; Fazio et al., 2013). The effect of temperature on blood hematological and biochemical indices have been studied in rainbow trout (*Onchorhynchus mykiss*) (Skov et al., 2011), goldfish (*Carassius auratus*) (Imanpoor et al., 2012), tilapia (*Oreochromis niloticus*) (Qiang et al., 2013), red spotted grouper (*Epinephelus akaara*) (Cho et al., 2015) and Atlantic cod (*Gadus morhua*) (Kunz et al., 2016) to clarify its importance. The hematological characteristics of many fish species have been examined to determine the normal ranges, and any variations in these ranges are indicative of pathophysiological trait (Gale et al., 2013). Although fish hematology is a promising valuable tool in the development of aquaculture systems, particularly its use for the detection of healthy individuals from diseased or stressed animals, progress in establishing normal range values for blood parameters has been impeded, and literature in this area is isolated and often incomplete (Segner et al., 2012; Fazio, 2018). These responses to stressors in fish are often characterized as primary, secondary, and tertiary (Ferguson et al., 2010) and involved in the activation of brain centers, resulting in the release of glucose and cortisol from steroid producing cells. The subsequent actions and effects of these hormones at blood and tissue levels may include a disturbance of the metabolic balance, which inhibits growth and suppresses blood hematological and biochemical parameters.

The newly developed TGGG hybrid is gaining popularity in Southeast Asian markets (Rimmer and Glamuzina, 2019), but explorations on health status responses to the temperature shock and diet variation of this species are scarce. This study was performed to evaluate the effect of temperature (22 °C, 26 °C, 30 °C, and 34 °C) and diets

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(commercial pellets or shrimp) on the hematological and biochemical responses and on serum cortisol and glucose concentrations in the TGGG hybrid grouper collected from local hatcheries in Banting, Selangor, Malaysia. The data obtained from this work could be used to monitor the physiological status of the TGGG hybrid which will ensure better management approach in grouper aquaculture.

2. Materials and methods

2.1. Sample collection and experimental setup

A total of 200 TGGG hybrid fish (body weight = 200 ± 15 g; length = 22.5 ± 1 cm) were used in this experiment. The fish were obtained from local hatcheries of Banting Selangor (2°49'0"N, 101°30'0"E) and immediately distributed randomly in three stocking tanks (tank size: 1.96 × 1.02 × 0.61 m, 1200 L) with running seawater and acclimated for 3 weeks. The fish samples were then randomly transferred to 40 experimental tanks (5 fish / tank), where the experiment was conducted for 60 days. The tanks had equal sizes and heights, with an area (length × width) of 0.6 m^2 and a height of 0.58 m. Twenty experimental tanks were randomly selected for the pellet diet, the twenty remaining tanks were for the shrimp diet. Five replicates were prepared at each temperature (22 °C, 26 °C, 30 °C, and 34 °C). The changes in temperature in the experimental groups were initiated at a rate of 1 °C day^{-1} by using a heater (E-JET heater 200 W, Penang, Malaysia) and a chiller (HS-28 A, 250–1200 L/H, Guangdong Hailea Grouph Co., Ltd., China) until the experimental temperature reached 22 °C (minimum) and 34 °C (maximum). During the acclimation and experimental period, the fish were divided into two groups, one group fed with commercial pellets (46% protein, 8% lipid & 7% carbohydrate; Marine 9982/84 Star feed, CP Group, Malaysia) or freshly thawed shrimp (58% protein and 8% lipid & 8% carbohydrate; *Acetes* sp.) in another group (Noor et al., 2018; Mazumder et al., 2019). Fish were fed twice daily (0900 and 1600 h) but were not fed 24 h prior to the experiment. The proximate composition of the experimental feeds was determined by using standard procedures; viz., protein (Raymont et al., 1964), lipid (Folch et al., 1957), and carbohydrate (Dubois et al., 1956). During this experimental period, samples were maintained on a 12 h light: 12 h dark photoperiod (Das et al., 2018).

2.2. Water quality parameters

The water quality parameters of temperature, salinity, and pH were monitored daily (Table 1). NH_3-N and total hardness were measured weekly in the morning before feeding, whereas temperature, pH, and salinity were determined in the morning at 0900 and monitored using a YSI 59 multiparameter water quality probe (Yellow Springs Instrument Company OH, USA). Total ammonia nitrogen (NH_3-N) and total hardness were measured via the salycilate method (HachTM method 8155) and titration by using a La Motte chemical test kit (Model WAT-DR) (Mazumder et al., 2018).

2.3. Blood component analysis

The experimental fish (22 °C, 26 °C, 30 °C, and 34 °C) were randomly collected from each experimental tank (1 fish / replicate tank) and anesthetized with α-methyl quinoline (Transmore®; Nika Trading, Puchong, Malaysia) (0.22 ml L^{-1} in 3 l of sea water as an anesthetic medium). Total length was measured to the nearest 0.01 cm with a measuring board, and weight was recorded to the nearest 0.01 g accuracy by using an electronic balance (Model KD-300KC) (Mazumder et al., 2016). The blood (~10 ml) samples were then immediately collected from the caudal aorta of each fish with a 2 ml sterile plastic syringe and transferred into two different tubes: one (Miniplast 0.5 ml, LP Italiana Spa, Milano) containing ethylenediamine tetraacetic acid (EDTA) (1.26 mg / 0.6 ml) as an anticoagulant agent and the other without EDTA for serum collection. The EDTA tubes with blood were used to determine the hematological profile. The collected blood samples were immediately subjected to haematological analysis. The blood was diluted with appropriated diluting fluids for RBC and WBC counts were determined using improved Neubauer haemocytometer and calculated (Blaxhall and Daisley, 1973). Replicated counts were made for each blood samples. Sahli's haemoglobinometer was used to estimate haemoglobin (HB) percentage (HB%). Haematocrit (HCT) was determined using micro haematocrit capillaries filled with blood and centrifuged at 8,700 × *g* for 5 min and expressed as percentage of total blood volume (Wintrobe, 1974). Mean corpuscular volume (MCV), mean cell haemoglobin (MCH) and mean cell haemoglobin concentration (MCHC) were calculated from the average values of HB% (Dacie and Lewis, 1984).

The second blood sample was left in an oblique plastic rack at room temperature for approximately half an hour to allow the blood to clot. The clotted blood was then centrifuged for 10 min at 12,000 rotations per minute (rpm) to isolate the serum, which was collected (800 µl) as a supernatant and stored into a clean and sterilized 1.5 ml sterile Eppendorf tube for further analysis (Peck et al., 2003). Parameters such as total protein (TP), albumin (ALB), globulin (GLB), alkaline/globulin ratio (A/G ratio), alkaline phosphatase (AP), aspartate aminotransferase (AST), and alanine transaminase (ALT) were subjected to biochemical analyses by using assay kits (Biosino Bio-technology and Science Inc, Beijin, China) (Almroth et al., 2015). Plasma cortisol concentration was measured through radioimmunoassay. Glucose, lactic acid, triglyceride (TRI), cholesterol (CHOL), bilirubin (BIL) and blood urea nitrogen (BUN) levels were analyzed using an automatic chemistry analyzer (Vitros DT60II, Vitros DTEII, DTSCII Chemistry System, Johnson & Johnson Clinical Diagnostics Inc., New York, USA).

2.4. Statistical analysis

The hematological and biochemical indices of the blood of fish and their cortisol levels on either pellet or shrimp diets, a two-factor factorial model was initially used. When significant temperature × diet interactions were encountered, the cell means were analyzed in a one-factor linear model. To determine differences ($P < 0.05$) among the experimental groups within each diet, Tukey *post-hoc* tests were used. Data were analyzed using MINITAB ver. 17 (StatSoft Inc., Tulsa, OK, USA) (De et al., 2016) and Microcal Origin 8.0 graphic software (OriginLab, Northampton) (Das et al., 2014).

Table 1
Water quality parameters (mean ± standard deviation) measured during the period of experiment.

Parameters	22 °C	26 °C	30 °C	34 °C
Temperature (°C)	22 ± 0.56	26 ± 0.14	30 ± 0.29	34 ± 0.29
Salinity (psu)	30 ± 0.45	30 ± 0.34	30 ± 0.36	30 ± 0.12
TH (mg L^{-1})	119.33 ± 22.33	108.07 ± 18.41	125.4 ± 16.49	97 ± 3.89
NH_3-N (mg L^{-1})	0.25 ± 0.20	0.25 ± 0.20	0.33 ± .24	0.42 ± 0.24
pH	7.47 ± 0.09	7.07 ± 0.31	7.27 ± 0.17	6.87 ± 0.17

Table 2
Blood haematological parameters (mean ± standard deviation) of TGGG in different temperatures and diets. Different superscript letters indicated significant different among treatments.

Parameters		RBC	WBC	TC	HG	PCV	MCV	MCH	MCHC
Diet									
	Shrimp	1.64 ± 0.05 ^{a}	132.78 ± 1.1^{a}	24.51 ± 2.04^{a}	8.025 ± 1.021^{a}	28.50 ± 2.33^{a}	169.25 ± 5.66^{a}	50.95 ± 2.77^{a}	30.25 ± 1.11 ^{a}
	Pellet	1.53 ± 0.05^{b}	128.02 ± 0.97^{b}	21.40 ± 1.11^{b}	6.915 ± 0.988^{b}	25.25 ± 1.47^{b}	149.90 ± 2.87^{b}	44.80 ± 2.04^{b}	28.05 ± 0.56 ^{b}
Temp.(°C)									
	22	1.35 ± 0.13^{c}	116.75 ± 3.37^{d}	16.41 ± 1.01^{d}	5.82 ± 0.57 ^{c}	23.9 ± 3.3^{b}	146.9 ± 5.1^{b}	43.3 ± 4.0 ^{c}	27.0 ± 2.5 ^{c}
	26	1.66 ± 0.05^{b}	136.29 ± 2.77^{b}	24.02 ± 2.33^{b}	7.94 ± 0.57 ^{b}	26.6 ± 3.1^{b}	167.7 ± 6.8^{a}	48.8 ± 3.7^{b}	29.6 ± 3.3 ^{ab}
	30	1.85 ± 0.03^{a}	141.23 ± 2.20^{a}	32.79 ± 4.55^{a}	9.80 ± 0.87^{a}	32.0 ± 4.1^{a}	173.0 ± 7.8^{a}	53.4 ± 4.4^{a}	31.2 ± 2.6^{a}
	34	1.48 ± 0.04^{c}	127.34 ± 3.21^{c}	18.62 ± 1.55^{c}	6.32 ± 0.34 ^{c}	25.0 ± 2.3^{b}	150.7 ± 4.9^{b}	46.0 ± 3.9 ^{bc}	28.8 ± 3.1 ^{bc}

3. Results

3.1. Blood ematological and biochemical parameters

Temperature and diet significantly affected the blood hematological parameters (Table 2; $P < 0.05$). No interaction occurred between temperature and diet except TC and WBC. The significantly highest hematological indices were RBC, WBC, TC, HG, PCV, and MCH ($P < 0.05$) at 30 °C compared with those at 26 °C, 22 °C, and 34 °C. Although MCV and MCHC were high at 30 °C, these indices were not significantly different ($P > 0.05$) from those at 26 °C; however, they were significantly different ($P < 0.05$) from those at 22 °C and 34 °C.

The biochemical indices obtained under the experimental temperatures and diets differed (Table 3). TP, ALB, and GLB were the highest at 30 °C, whereas ALT, AST, and alkaline phosphate were the highest at 34 °C. ALB showed a similar trend; that is, it increased by 26.1% from the lowest value at 22 °C with the pellet diet to the value at 30 °C with the shrimp diet. However, GLB levels decreased by 15.7% from the value at 22 °C in the pellet-fed group to the value at 30 °C (from 16.6 g L^{-1} to 14.0 g L^{-1}) in the shrimp-fed group. Variations in TP, ALB, and GLB were not significant ($P > 0.05$) in any of the tested temperature. The ratio of A/G increased significantly ($P < 0.05$) by 49.4% (1.66 at 22 °C for the pellet-fed group to 2.483 at 30 °C for the shrimp-fed group) from 22 to 26 and 30 °C. Similarly, AST and ALT levels were the highest in the group at 30 °C. These parameters increased at 22 °C to 30 °C, but they decreased as temperature further increased to 34 °C. However, these variations were not significant ($P > 0.05$). Conversely, a significant interaction was found between temperature and diet in terms of ALT ($P < 0.05$). Unlike the hematological parameters except globulin, the values of the biochemical parameters were higher in the shrimp-fed fish than in the pellet-fed fish. However, no significant difference ($P < 0.05$) was observed in the diet variations in any of the temperatures except AKP, whose variations were significant at 26 °C ($P < 0.05$).

3.2. Glucose and cortisol level

Fig. 1 demonstrates the effects on glucose concentrations following different temperatures and diets at the beginning and at the end of the experiment. Glucose concentration was not significantly different at the initial time of the experiment. Glucose concentration remained the lowest in fish fed with the shrimp diet (26 ± 10 mg dL^{-1}) at 26 °C, whereas the significantly highest glucose was found at 22 °C. The fish fed with shrimp (100.4 ± 9.5 mg dL^{-1}) and pellet (108.3 ± 18.8 mg dL^{-1}) did not significantly differ. This outcome was supported by a significantly higher level of serum cortisol at 22 °C. The fish fed with shrimp (0.2 ± 0.1 ng mL^{-1}) and pellet (0.2 ± 0.1 ng mL^{-1}) did not significantly vary. Conversely, the lowest cortisol was found in fish fed with shrimp at 26 °C (0.5 ± 0.1 ng mL^{-1}) (Fig. 2). No significant differences were observed in both diets at all temperatures.

4. Discussion

Most blood hematological indices were higher at 30 °C than those at 22 °C, 26 °C, and 34 °C. A decrease in the RBC of the TGGG hybrid grouper is characterized by the smallest erythropoiesis at low temperatures. Similar observation has been documented in red spotted grouper (*Epinephelus akaara*) (Park et al., 2016). As temperature increases, oxygen absorption by RBC decreases. Thus, the body compensates this reduction by increasing the amount of RBC. Hb level is also connected with the number of RBC. When the number of RBC decreases, the Hb level decreases. A decrease in the trend of Hb at a low temperature has been reported in red spotted grouper (*E. akaara*) and Malabar grouper (*E. malabricus*) (Lin et al., 2012; Cho et al., 2015). A decrease in Hb can be a result of blood osmoconcentration as shown by an increase in plasma osmolarity. The Hb in this study is within the range of 37.6%–53.23%; a low Hb is associated with fish of low activity at 22 °C and 34 °C. Rambhaskar and Rao (1987) reported similar results in Indian mackerel (*Rastrelliger kanagurta*).

WBC is another key cell component in fish blood and involved in immune responses, although some authors suggested that fish TC also participates in defense mechanisms via their phagocytic ability (Stosik et al., 2001). WBC levels vary in terms of environmental temperature and nutritional state (Tavares-Dias et al., 2002; Tort, 2011). The WBC levels in this study were the highest at 30 °C (15.38% ± 3.13%) and the lowest at 22 °C. The WBC and TC decrease at 22 °C and 34 °C, thereby indicating a weakened defense (immunosuppression) and delay clotting when a fish is injured in the new environment, as reported in other fish

Table 3
Blood biochemical parameters (mean ± standard deviation) of TGGG in different temperatures and diets. Different superscript letters indicated significant different among treatments.

Parameters		TP	ALB	GLB	AKP	AST	ALT
Diet							
	Shrimp	40.35 ± 3.43^{a}	14.2 ± 2.3^{a}	23.60 ± 2.1^{a}	99.1 ± 4.5^{a}	46.25 ± 5.8^{a}	37.20 ± 14.03^{a}
	Pellet	38.80 ± 4.42^{a}	13.4 ± 3.7^{a}	25.25 ± 3.2^{a}	95.5 ± 5.0^{a}	41.50 ± 6.2^{a}	29.85 ± 12.33 ^{b}
Temp. (°C)							
	22	23.7 ± 11.5^{b}	8.2 ± 5.1^{c}	15.4 ± 6.9^{b}	80.0 ± 8.8^{c}	27.3 ± 8.4^{b}	12.6 ± 1.4^{d}
	26	34.1 ± 10.1^{b}	12.3 ± 4.7^{bc}	21.8 ± 5.2^{b}	119.7 ± 19.1^{a}	61.1 ± 18.8^{a}	53.6 ± 14.7^{a}
	30	53.9 ± 4.3^{a}	15.7 ± 4.9^{ab}	25.6 ± 9.6^{ab}	88.8 ± 13.7^{bc}	35.9 ± 7.6^{b}	26.5 ± 2.1^{c}
	34	46.6 ± 5.2^{a}	19.0 ± 5.5^{a}	34.9 ± 9.0^{a}	100.7 ± 12.4^{b}	51.2 ± 10.0^{a}	41.4 ± 13.7 ^{b}

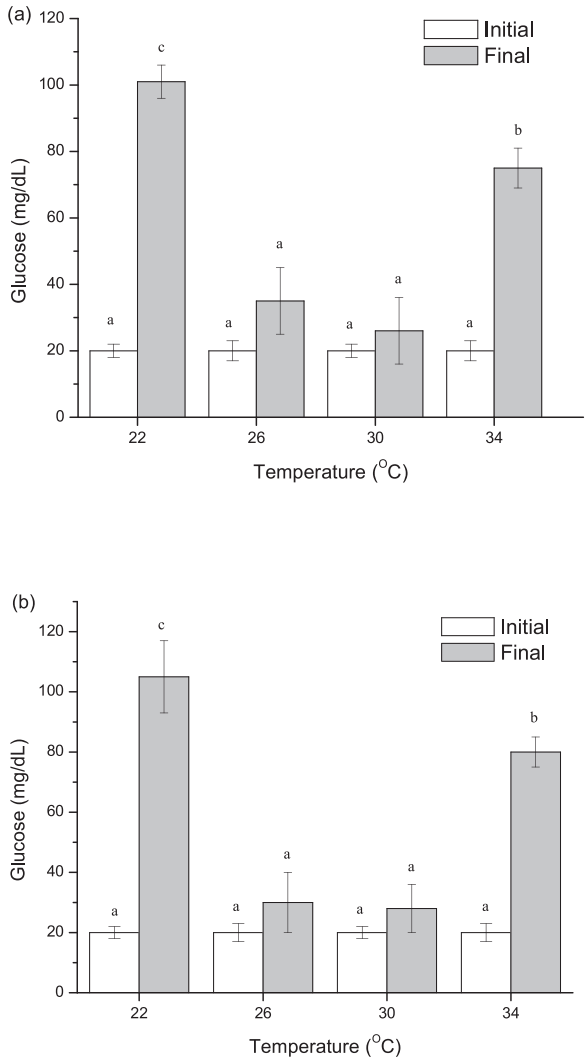

Fig. 1. Plot of mean ± standard deviation of glucose concentrations with respect to temperature, recorded on initial and final time of experiment in (a) shrimp fed and (b) pellet fed fish. White bars represent initial time and grey bars represent final time of experiment. Different superscript letters indicated significance difference among the treatments ($P < 0.05$) at the same sample time point.

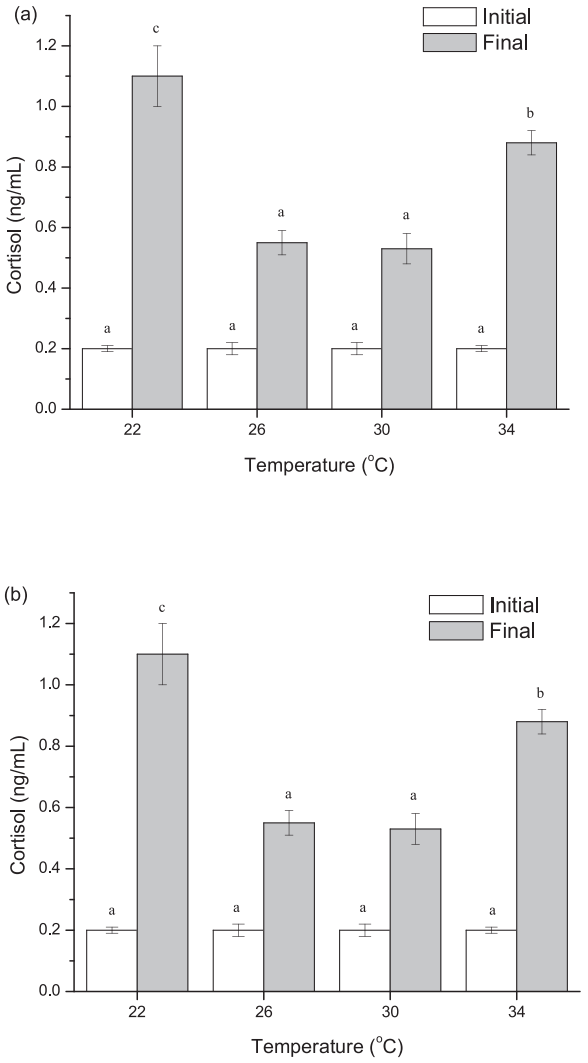

Fig. 2. Plot of mean ± standard deviation of cortisol concentrations with respect to temperature, recorded on initial and final time of experiment in (a) shrimp fed and (b) pellet fed fish. White bars represent initial time and grey bars represent final time of experiment. Different superscript letters indicated significance difference among the treatments ($P < 0.05$) at the same sample time point.

(Fazio et al., 2013). The differences in the WBC count at the four temperatures and the prepared diets in this study did not arise from the different physicochemical parameters of water, body size of fish, and stress (Zhou et al., 2009). This study showed an increase in the WBC of the fish fed with a shrimp diet compared with that of the pellet diet (Table 2) possibly because the fish fed with shrimp consequently consumed more protein than those with the pellet diet.

The hematocrit (Hct) value in this study was within the range of 23%–35%. Hct values are usually between 20% and 35% and rarely exceed 50% (Clark et al., 1979). Cho et al. (2015) presented similar results on red spotted grouper (*E. akaara*) whose Hct increases at a decreasing temperature (15 °C). Hct is a measure of the cellular fraction of blood and common stress indicator in fish. A similar increase was also observed in the TGGG hybrid grouper (Table 2).

In this research, RBC, Hb, Hct, PC, and WBC significantly changed with diet. This has been suggested by a study of introducing fungi as feed meal for kelp grouper (*E. bruneus*) juveniles by which does not significantly affect RBC counts but does not significantly improve Hct (Harikrishnan et al., 2012). Indeed, Hct and Hb concentrations in animals fed with protein-deficient diets significantly decrease. Hct can be adopted as a tool in the aquaculture industry for the routine monitoring of anemia in orange-spotted grouper (*E. coiodes*) (Huang et al., 2018).

The TP concentrations decreased as temperature increased (Table 3). The increased concentrations of TP can be caused by structural liver alterations, thereby reducing aminotransferase activity, decreasing deamination capacity, and impairing the control of fluid balance (Coz-Rakovac et al., 2005). However, the differences in blood biochemical parameters found in this study were attributed to the different temperatures and diet; the ranges of serum biochemistry can be influenced by many biotic and abiotic factors, such as water temperature, seasonal pattern, food, age, and sex of fish (Jawad et al., 2004). Variations in biochemical blood parameters may be due to different biochemical metabolic mechanisms at varied feeding temperatures and diets (Adeyemo et al., 2003; Subhadra et al., 2006). The possible importance of increased serum protein as a fuel for tissues during osmotic acclimation has not been addressed yet, but this observation may be related to a metabolic reallocation of energy resources once carbohydrate stores have been mobilized. Studies on the hematological and biochemical changes induced by replacing fish meal with plant protein in common carp (*C. carpio*) have indicated that experimental diets have

significantly different TP levels. AST and ALT found respectively in the hepatocytes and cardiomyocytes of fish play important roles in protein metabolism (Sadauskas-Henrique et al., 2011). When liver and myocardial cells are damaged or when their permeability increases, AST and ALT are released into the blood, resulting in an increased blood transaminase activity. The activities of serum AST and ALT can be used to monitor the health status of fish (Javed and Usmani, 2019). In the present study, AST and ALT at different temperatures and diets changed, but the values remained in the normal range, indicating that fish are in good condition (Tavares-Dias et al., 2000).

Data for comparing the obtained haematological and biochemical indices in the present study are insufficient, making it a challenge to conclude with high degree of confidence on the best condition of TGGG hybrid grouper aquaculture. However, these results can be considered as baseline data for this species. The observed increase in the values of the hematological parameters post-bleeding is difficult to explain, but hematological values in fish are highly variable (Sadauskas-Henrique et al., 2011). Establishing normal hematological values in fish is challenging, and these values are highly relative; moreover, no precise readily defined division exists between normal and abnormal (Fazio, 2018).

In general, serum glucose and cortisol have been widely accepted as indicators of stress measurement in fish because they increase after fish are exposed to physical stressors (Davis and Parker, 1990). Circulating cortisol levels in the blood are usually measured to determine the stress level of individual fish (Dolomatov et al., 2013). Many researchers evaluated the change in water quality as a stressor to fish and monitored its effects on blood chemistry, including serum cortisol (Cowan et al., 2017) because water quality stress, not diet type, increases serum cortisol in sunshine bass (*Morone chrysops* × *Morone saxatilis*) (Davis, 2004). On the whole, it is affirmed that serum cortisol is a good indicator of stress in many fish species (Cowan et al., 2017).

5. Conclusion

As important considerations for analyzing the health status of fish, physicochemical factors and diet influence the blood parameters and serum of fish. In this research, TGGG fed with shrimp at 26 °C demonstrated the best blood and stress condition compared to those with other temperatures and diets. The variations in hematological and biochemical parameters supported by serum glucose and cortisol evaluations due to different temperatures and diets emphasize that changes in blood characteristics are important indices for monitoring the management of fish physiology. Therefore, establishing a baseline of information for fish blood and serum profile as a monitoring tool for aquaculture systems may improve the welfare and production of the TGGG hybrid grouper. Such information would help in the early detection of stressful and other disease conditions that might interfere with the performance of the species in some vital cultural operations, such as induced breeding and nutritional programs. These data would also be useful for fish biologists in assessing fish health and monitoring stress response to develop healthy management mechanisms and support the rapid progress in the aquaculture industry in terms of the hematology, biochemistry, and glucose and cortisol analysis of fishes.

Declaration of Competing Interest

The authors have declare that no conflict of interests exist.

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