

Effect of water temperature on the physiological responses in *Betta rubra*, Perugia 1893 (Pisces: Osphronemidae)

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Abstract Water temperature is a limiting factor in fish health and plays a crucial role, especially in endemic species that are more sensitive to ambient temperature changes. The objective of the present study was to examine the effects of temperature on growth patterns, survival, blood glucose, gill histology, and erythrocyte cell abnormality of *Betta rubra*, an endemic species in Aceh and Northern Sumatra, Indonesia. The fish sample was collected from Nagan Raya, Aceh Province, Indonesia. The fish were acclimatized for three days prior to experimental trials at five temperature levels, 24, 26, 28, 30 and 32 °C for 14 days. Fish were taken randomly from every treatment to measure blood glucose levels and gill samples were taken at the start and end of the experiment. The results showed the highest survival at a temperature of 28 °C (83.33%). The lowest blood glucose level was also found at a temperature of 28 °C. In addition, an increase and decrease in temperature exceeding 28 °C caused gill damage. Higher temperatures caused an increase in the abnormality of erythrocyte cells, with the highest percentage of abnormality found at 32 °C. Overall, this study confirmed that exposure to lower and higher temperature than the optimal is stressful to *B. rubra*.

Keywords Gill abnormality . *Betta rubra* . Blood glucose . Ambient temperature

Introduction

Indonesia is one of the most biodiverse countries in the world. According to Fishbase (2019), at least 4,878 species of fish have been recorded from Indonesian waters, of these 1,258 species are freshwater fish. However, these numbers are underestimated since many locations have never been explored and therefore many new species are being described every single year. Sumatera island is one of the most biodiverse regions in Indonesia (Muchlisin and Azizah 2009). According to Kottelat et al. (1993) there were 272 species of freshwater fish reported from Sumatra, including 30 species endemic to the island. Wargasmita (2002) reported 589 species of freshwater fish in Sumatra, including 58 endemic species with 14 of these threatened by ecological perturbation and climate change (Booth and Evans 2011; Oczkowski et al. 2015;

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Kano et al. 2016).

Global warming that triggers an increase in the average temperature of the air temperature (Nguyen et al. 2011; Lineman et al. 2015). Based on the Climatology, Meteorology and Geophysics Agency of Republic of Indonesia, the earth temperature has been increasing approximately 0.03 °C annually (Bmkg 2019). Climate change may affect fish directly through at least two mechanisms. Firstly, acidification occurs as atmospheric CO₂ increases (Cole and Prairie 2009), a process which threatens the fish populations (Orr et al. 2005; Doney et al. 2009; Pachauri et al. 2014). Secondly, increases in water temperature associated with climate change negatively impact fish communities, for example through changes in species composition (Caldeira and Wickett 2003; Xu et al. 2009; Bezuijen 2011; Oczkowski et al. 2015), distribution and abundance (Gupta et al. 2012; Kjelland et al. 2015).

Fish are poikilothermic animals which adjust their body temperature to the ambient water temperature (Bowden 2008 ; Abram et al. 2017; Frick et al. 2018). However, there are limits to fish physiological adaptation to changes in ambient temperature, and therefore the water temperature can be a limiting factor for fish health (Viadero 2005; Simpson et al. 2013). Tang et al. (2017) and Nyanti et al. (2018) reported that changes in water temperature and pH can cause stress, decrease feeding activity, reduce growth and survival rates and lead to erythrocytic abnormalities such as echinocytic and elongated shape (Ashaf-Ud-Doula et al. 2019; Shahjahan et al. 2019; Shahjahan et al. 2020). Moreover, the changes in water temperature also cause damage to gill cells (Mohammadi et al. 2019). Indeed, this impact is highly significant in endemic species (Leuven et al. 2011; Davis et al. 2019). According to Ficke et al. (2007), endemic fish typically have narrow tolerance to environmental changes including temperature, pH and dissolved oxygen, and therefore are very susceptible to extinction due to climate change.

Betta rubra, locally referred to as *cupang* fish, is one of the endemic fish species found in Aceh waters. This species is distributed from the Southwestern part of Aceh to Sibolga in North Sumatra (Hui 2013). *Betta rubra* has an important role both ecologically and economically. This species is a highly economically important ornamental fish and plays a crucial trophic role in the freshwater ecosystem. Besides, *B. rubra* is an invaluable germplasm, because of its endemism in Sumatra island. However, the population of *B. rubra* has been decreasing over the years due to habitat destruction, over-exploitation of the wild population, introduction of alien fish species and climate change (Muchlisin 2008). Therefore, this species has been listed as an endangered species in the IUCN Redlist database (Low 2019)

Research on *B. rubra* is scarce. The two documented studies in the literature have been limited to the description and taxonomic status of the *B. rubra* (Schindler and Van Der Voort 2011; Hui 2013). However, studies on the physiological response due to temperature change have never been published. This information is important to predict the effects of climate change and to plan a better mitigation strategy for this species. Therefore, the objective of the present study was to examine the effects of water temperature changes on the growth performance, survival, gill morphology and blood profiles of *B. rubra*, an endemic species in Sumatra.

Materials and methods

Experimental fish

Adult *B. rubra* with mean bodyweight of 0.5±0.10 g and mean total length of 3.90±0.30 cm were used in this experiment. The fish were collected from Nagan Raya, Aceh, Indonesia during the dry season (27.8-28.7 °C) using scoop nets and acclimatized for three days in the Laboratory of Ichthyology, Faculty of Marine and Fisheries, Universitas Syiah Kuala, Banda Aceh. During acclimatization, the fish were fed twice a day up to satiation with commercial feed (Hi-Pro-Vite 888 with 42 % crude protein).

Experimental design and research procedure

A completely randomized experimental design with one tested factor (univariate) was used in this study. The tested factor was differences in water temperature at five levels, modified from Hassanen et al. (2014), namely; 24, 26, 28, 30 and 32 °C. Each temperature treatment was conducted with three replications. During the procedure, a fish was taken randomly one at a time from the acclimatized tank



and measured for initial length and weight, then stocked into 15 plastic containers (45 cm x 35 cm x 30 cm in size) with 5 L of water at a stocking density of 15 fish per container (Niazie et al. 2013). The experiment was conducted in the laboratory temperature-controlled room. The water temperature in the containers was adjusted to room temperature (24 °C) with a heater (Amara HT-100). The temperature was increased gradually at 1 °C per 12 hours until it reached the testing point (Shahjahan et al. 2019). The fish were reared at the treatment temperature for 14 days (Shahjahan et al. 2020).

Weight gain and survival rate analysis

Weight gain was calculated as follows: $Wg = Wt - Wo$, where Wg = weight gain (g), Wt = average body weight of fish at the end of the experiment (g), Wo = average body weight of fish at the start of the experiment (g). The survival rate (SR) of the fish was calculated based on Muchlisin et al. (2016) and Muchlisin et al. (2017) as follows: $Survival (\%) = (No - Nt)/No \times 100$, where No is total fish at the start of the experiment, Nt is total fish mortality during the experiment.

Blood glucose analysis

The blood glucose was examined on five occasions: firstly, in the field when the fish was sampled; secondly, upon reaching the laboratory from the field; thirdly, after three days acclimated in the laboratory; fourthly, at day-7 of the experiment; and fifthly, at day-14 of the experiment. The blood was collected from three randomly sampled fish from each treatment. The fish was anesthetized using MS-222 and euthanised. Immediately after collection of blood from caudal peduncle of experimental fish, blood glucose level was estimated using glucose strips by Easy-Touch GCU.

Erythrocyte abnormality analysis

For erythrocyte abnormality analysis, each blood sample was smeared onto a clean glass slide and dried at room temperature for 10 min. The sample was then fixed using 98% methanol for another 10 min. After a further 10 min the sample was stained using 5% Giemsa solution, and then the sample was washed using distilled water and dried at room temperature for 24 h. The sample was observed using a stereomicroscope (Primo Star Zeiss, Germany) with 100x objective lens. The erythrocyte abnormality was observed by random sampling of 900 erythrocyte cells. The abnormality was categorized into two categories; erythrocytic nuclear abnormalities (ENA) and erythrocytic cellular abnormalities (ECA) (Shahjahan et al. 2018). The ENA is classified as: binucleated and nuclear bridge while ECA is classified as: fusion, teardrop, and twin shaped cells (Carrasco et al. 1990).

Histological analysis of gill morphology

Gill morphology was observed using histological analysis after the experiment. Four fish samples were taken randomly from each treatment and the fish anesthetized using MS-222 and euthanised (Nugroho et al. 2017). The mouth was then dissected from the *cavum oris* to the lower area of the operculum. The gill arch was separated and the whole gill was removed from the gill cavity and fixed in Bouin solution for 24 h. The second gill arch was dehydrated in an increasing alcohol series and then cleared using a combination of xylene and alcohol, embedded in paraffin and sectioned (4-5 µm) using a microtome (HM 325 Rotary Microtome, Thermo Scientific™). The sections were attached to a glass slide and dried in a stove (37 °C) for 24 h and followed at 60 °C for one hour. The sample was stained with Ehrlich hematoxylin-eosin (Muchlisin et al. 2010). The gill morphological abnormalities were observed in primary lamella and secondary lamella using a stereo microscope (Primo Star Zeiss, Germany, 100x objective lens). The criteria for defining gill cell abnormalities were based on Bernet et al. (1999).



Data analysis

The data on blood glucose, Erythrocyte Abnormality and survival rate were subjected to one-way Analysis of Variance (one-way ANOVA) followed by Duncan's multiple range test with 95% confidence limits. Data on gill cell abnormality were analyzed descriptively.

Results

Weight gain and survival rate

The experimental fish exposed at 28 °C showed a weight gain of 0.02 g, while fish exposed to 26 °C did not gain weight and in fact even lost weight during the experiment (Table 1). The highest survival rate was recorded for treatment at 28 °C (83.3%), while the lowest survival was recorded at 24 °C (0%, all fish died). The mortality starts to occur at day-1 and all fish died at day-6 at 24 °C. At day-14, the lowest survival rate was found for fish reared in 26 °C (3.33%). The rate increased to 83.33% for fish reared at the water temperature of 28 °C, then decreased gradually at a temperature above 30 °C (Table 1).

Table 1 Growth and survival rates of *Betta rubra* exposed to different water temperatures for 14 days

Parameter	Exposed temperature				
	24 °C	26 °C	28 °C	30 °C	32 °C
Initial weight (g)	0.48±0.02	0.50±0.01	0.50±0.02	0.49±0.02	0.49±0.02
Final weight (g)	-	0.33	0.52±0.03	0.62±0.03	0.50±0.02
Weight gain (g)	-	-0.17	0.02	0.13	0.01
Survival rate (%)	0	3.33±0.33 ^a	83.3±0.33 ^c	70.00±5.77 ^{bc}	53.33±13.33 ^b

Note: Mean±SD in same row with different superscript are significantly different (P< 0.05)

Table 2 Blood glucose concentration (Mean±SD) of *Betta rubra* in the field, laboratory and post acclimatization

Tank No.	At the field (<i>in situ</i>)	At laboratory post transportation	At laboratory three-days post acclimatization
1	1.40±0.0 mmolL ⁻¹	4.05±0.07 mmolL ⁻¹	2.80±0.10 mmolL ⁻¹
2	1.50±0.14 mmolL ⁻¹	4.10±0.0 mmolL ⁻¹	2.75±0.05 mmolL ⁻¹
Average	1.45±0.10 mmolL ⁻¹	4.08±0.50 mmolL ⁻¹	2.77±0.07 mmolL ⁻¹

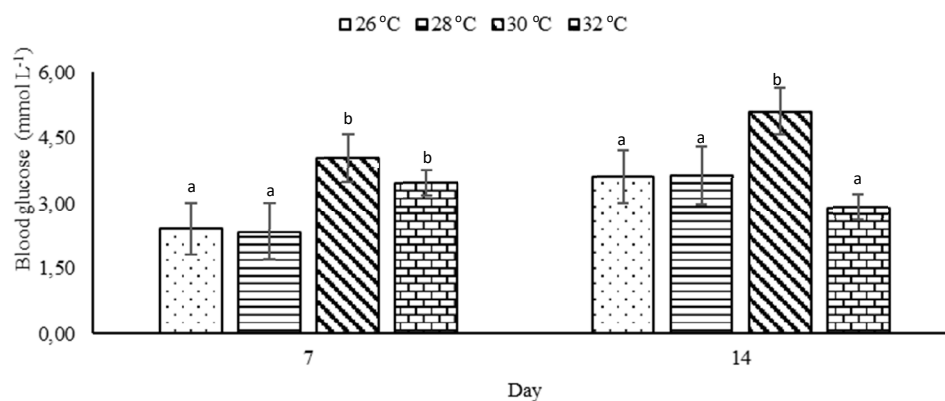


Fig. 1 Changes in blood glucose concentration post-exposure to different temperatures. Values accompanied by different letters indicate statistically significantly difference (P< 0.05).



Blood glucose

The blood glucose levels of the fish in the field just after sampling was 1.4-1.5 mmolL⁻¹. These values increased to 4.05-4.1 mmolL⁻¹ post transportation from the field to the laboratory and then decreased to 2.75-2.80 mmolL⁻¹ after acclimation for three days (Table 2). We assume that the fish had already adapted to the laboratory conditions at the initiation of the experiment. All the fish at the treatment temperature of 24 °C died before day-7, and therefore there is no data from this treatment. At day-7, the blood glucose ranged from 2.23 mmol L⁻¹ to 4.03 mmol L⁻¹ and at day-14 the blood glucose ranged from 2.90 mmol L⁻¹ to 5.10 mmol L⁻¹, the highest concentration of blood glucose was observed at the temperature of 30 °C (Figure 1).

Erythrocyte cell abnormalities

Seven types of erythrocyte abnormalities were observed at the end of the experiment (Figure 2). Erythrocyte abnormalities of echinocytic was highest at 32 °C, and was significantly different from other treatments ($P < 0.05$). The fusion, elongated shape, and twins were also highest at 32 °C. These values were significantly different from 28 °C, but were not significantly different from 30 °C ($P > 0.05$). Binucleated, nuclear bridge and tear-drop shape were not significantly different among the treatments ($P > 0.05$) (Table 3).

Gill abnormalities

Based on histological analysis, four types of gill abnormalities were identified, namely; shortening of secondary lamella (SH), fused secondary lamella (F), aneurysm (A), and blood congestion (CB) (Figure 3). Secondary fusion was observed at 26 °C, while congested blood primer lamellae were recorded at 30°C and 32 °C. At 28 °C, the gill histology showed normal conditions without any abnormalities.

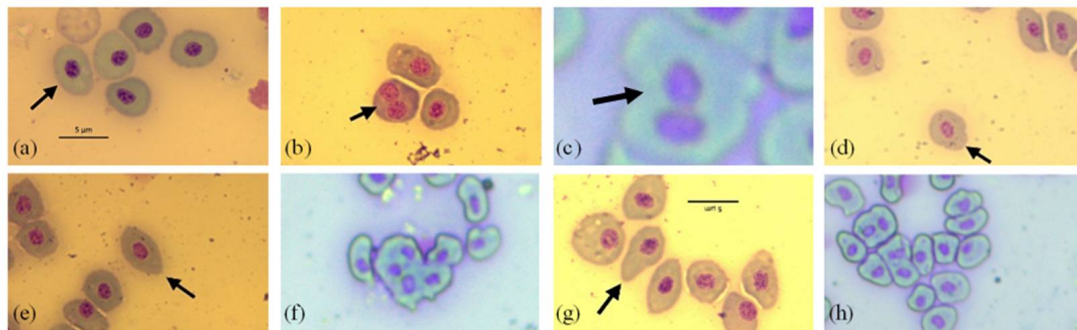


Fig. 2 Erythrocyte abnormality (a) regular cell, (b) binucleated, (c) nuclear bridge, (d) echinocytic, (e) elongated shaped, (f) fusion, (g) tear drop shaped, and (h) twin.

Table 3 Erythrocyte abnormalities of *Betta rubra* after 14 days exposed at different temperatures

Temp.	Binucleated (%)	Nuclear bridge (%)	Echinocytic (%)	Fusion (%)	Elongation shaped (%)	Tear drop shaped (%)	Twins (%)
28 °C	0±0 ^a	1.33±0.19 ^a	2.22±0.22 ^a	1.78±0.29 ^a	1.67±0.19 ^a	0.89±0.48 ^a	1.56±0.29 ^a
30 °C	0.11±0.11 ^a	1.56±0.29 ^a	5.89±0.48 ^b	2.56±0.29 ^{ab}	2.56±0.59 ^{ab}	2.00±0.19 ^a	2.67±0.19 ^b
32 °C	0.11±0.11 ^a	2.22±0.29 ^a	8.89±0.40 ^c	3.89±0.59 ^b	4.00±0.51 ^b	2.00±0.19 ^a	3.44±0.29 ^b

Mean±SD followed with different superscript in the same column are significantly different ($P < 0.05$).



Discussion

The current study revealed that *B. rubra* grows optimally at a temperature of 28 °C. Previous studies in the Siamese fighting fish, *B. splendens* showed that the best maintenance temperature range was 20-30 °C (Reyes-Bustamante and Ortega-Salas 2002). Our study showed that the survival rate of *B. rubra* decreased gradually when the water temperature rose from 28°C to 30 °C and 32 °C while there was 100% mortality within seven days at 24 °C. This study indicates that *B. rubra* cannot tolerate decreasing or increasing temperatures.

Several studies have reported that temperature is an abiotic factor that most influences the survival and distribution of fish (Kestemont et al. 2003; Martínez-Álvarez et al. 2005). At low temperatures, fish cannot produce enough heat for their bodies to tolerate the thermal conditions (Acharya and Mohanty 2014; Giannetto et al. 2014; Maisano et al. 2016). At temperatures of 26°C, 30°C and 32 °C, decreases in the body weight of fish were observed at the end of the experiment. In contrast, the body weight increased in fish reared at 28 °C. However, changes in temperature that exceed the tolerance limit trigger stress which can have negative impacts on fish (Dominguez et al. 2004), for example; stunted growth in meagre, *Argyrosomus regius* (Chatzifotis et al. 2018), late gonad maturation in Atlantic salmon, *Salmo salar* (King and Pankhurst 2007) and disease susceptibility in blueback salmon, *Oncorhynchus nerka* (Fish and Rucker 1945), inhibited growth, even death in Bull trout, *Salvelinus confluentus* (Selong et al. 2001), and negatively impacts fish metabolism in Brond snout, *Chondrostoma regium* (Mohammadi et al. 2019).

Hastuti et al. (2003) explained that the mechanism underlying the increase in blood glucose levels begins when the receptor organ receives a stressor, then this information is conveyed to the hypothalamic by the nervous system. The hypothalamus secretes the Corticoid Releasing Factor (CRF) which stimulates the pituitary gland to secrete Adrenocorticotrop hormone (ACTH), Melanophore-Stimulating hormone (MSH) and β -endorphin (β -End). These hormones regulate inter renal cells to secrete the cortisol hormone. The presence of the cortisol triggers an increase in the production of enzymes involved in gluconeogenesis. This will have an impact on increasing blood glucose which is an early indicator of fish experiencing stress (Mommsen et al. 1999; Beyea et al. 2005; Islam et al. 2019). This mechanism is linked to energy demand for maintaining the metabolic activity in tissue cells (Deslauriers and Kieffer 2012).

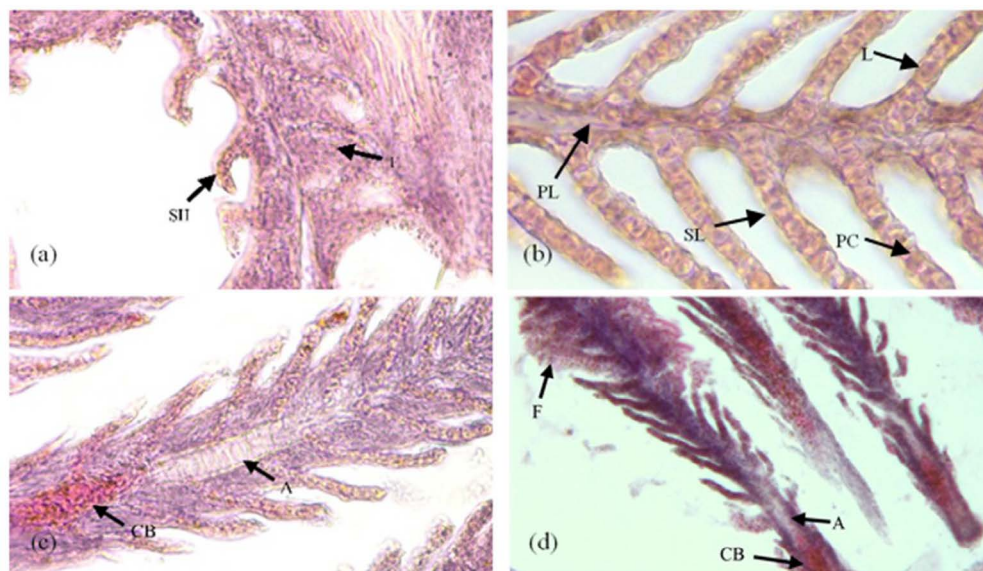


Fig. 3 Gill abnormalities of *Betta rubra* (a) at 26 oC showed shortening of secondary lamella (SH) and fusion of secondary lamella (F); (b) at 28 oC, the gill histology showed normal conditions, PL: primary lamella, SL: Secondary lamella, PC: Pillar cell, L: Lacuna; (c) at 30 oC blood congestion (CB); and (d) at 32 oC blood congestion (CB), fusion (F), and aneurysm (A).



According to Polakof et al. (2012), the average blood glucose level in Perciformes fish is about 3.7-4 mmolL⁻¹; therefore the blood glucose levels of *B. rubra* fish passed the tolerance limit except at treatment 28 °C. This indicates that *B. rubra* is physiologically disturbed due to stress. At 26 °C the fish also experienced an increase in blood glucose levels by up to 140% (3.6 mmolL⁻¹). This is an indication that the fish was experiencing stress due to low temperature shock, while the average level of glucose in wild *B. rubra* (no stress conditions) is 1.5 mmolL⁻¹. Similar results were reported in previous studies on several species such as in Channel catfish, *Ictalurus punctat* (Strange 1980), rohu, *Labeo rohita*, common carp, *Cyprinus carpio* (Chatterjee et al. 2004), Hong Kong grouper, *Epinephelus akaara* (Cho et al. 2015) and striped wallago catfish, *Wallago leeri* (Tang et al. 2017). However, the blood glucose decreased to 2.23 mmolL⁻¹ at a temperature of 28 °C; not much deviated from normal level of blood glucose of the species in the wild. The survival rate was also higher (83.33%). The highest blood glucose concentration (above 4.0 mmol L⁻¹) was found at 30 °C, indicating that the fish was in a stressed condition (Cech and Brauner 2011). The increase in glucose level in this treatment could be caused by the alteration of glycogen to glucose to meet the increased energy demand needed to reduce stress due to temperature shock (Xie et al. 2017). However, in *B. rubra*, the negative impact of temperature decrease is more severe than temperature increase as recorded in this study. Tang et al. (2017) reported optimum blood glucose level in the striped wallago, *Wallago leeri* maintained at 29 °C, while an increase or decrease of 3 °C of water temperature impacted blood glucose levels. A similar finding was also reported in the pacu, *Piaractus mesopotamicus* reared at a temperature of 18 °C to 30 °C (Pinto et al. 2019).

Several studies have shown that the effects of thermal stress can cause changes in hematological parameters in response to hypoxia or anoxia (Carvalho and Fernandes 2006). Erythrocyte abnormalities such as elongated, fusion, tear-drop shaped and twin shaped cells in fish exposed to high temperatures has been attributed to increased lipid peroxidation in erythrocytes as recorded in this study. Echinocytic is caused by erythrocyte membrane lipid solubility which leads to apoptosis (Walia et al. 2013), while binucleated is because of failure of cytokinesis at the stage of cell nucleus division but which is not followed by cytoplasmic division (Pera and Schwarzacher 1968). The results of this study indicate that in general, the abnormality of red blood cells increases with an increase in temperature. Similar findings were also reported in the rohu, *Labeo rohita* (Ashaf-Ud-Doulah et al. 2019), common carp *Cyprinus carpio* (Shahjahan et al. 2020), zebrafish *Danio rerio* (Shahjahan et al. 2018) and striped catfish *Pangasianodon hypophthalmus* (Islam et al. 2019).

The zero to low survival rates at temperature of 24 °C was probably due to the low temperatures affecting metabolic decline (Mohammadi et al. 2019), which is associated with gill damage (Leino and McCormick 1993; Mohammadi et al. 2019). Therefore, the decrease in metabolism, and subsequent gill damage caused most of the fish to die. This phenomenon was also found at higher temperature of 32 °C.

Conclusion

Betta rubra is unable to tolerate changes in the ambient water temperature of its habitat, causing an increase in blood glucose levels, abnormal red blood cells, and damage to the gill cells, and subsequently resulting in mortality.

Conflict of interest The authors declare that they have no conflict of interest.

Authors' contributions FMN and ASB: Conceptualization, Data Curation, Formal Analysis, Investigation, Methodology, Resources, Software, Validation, Visualization, Writing – Original Draft Preparation; KE, UT and AAM: Conceptualization, Methodology, Supervision, Writing – Original Draft Preparation; MNS, MW, NF and SR: Conceptualization, Formal Analysis, Supervision, Writing – Original Draft Preparation; MZA: Conceptualization, Funding Acquisition, Supervision, Writing – Review & Editing. All authors read and approved the final manuscript.

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