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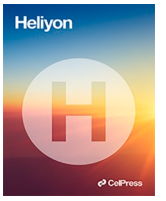

Research article

Impact of rising temperature on physiological and biochemical alterations that affect the viability of blood cells in American bullfrog crossbreeds

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ABSTRACT

The study aimed to examine the impact of increasing environmental temperatures on physiological changes, oxidative stress, nitric oxide production, total antioxidant capacity, and blood cell viability in American bullfrog crossbreeds. Frogs and frog blood cells were exposed to temperature ranges of 25–33 °C and 25–37 °C, respectively. Physiological parameters (body temperature, pulse rate, ventilation rate, and oxygen saturation) and biochemical parameters (total antioxidant power, hydrogen peroxide, malondialdehyde, nitric oxide, and mitochondrial activity) were measured at every 2 °C increment. Results showed that body temperature rose with increased environmental temperature ($P < 0.05$). Pulse rates at 33 °C were higher than those at 25–31 °C ($P < 0.05$). Ventilation rates at 31 °C exceeded those at 25 °C and 27 °C ($P < 0.05$). Oxygen saturation levels remained stable at 25–33 °C ($P > 0.05$). Total antioxidant power at 25 °C was greater than at 27–37 °C ($P < 0.05$). Hydrogen peroxide levels at 27 °C were higher compared to 25 °C and 31–37 °C ($P < 0.05$). Malondialdehyde levels at 25–33 °C were higher than at 35 °C and 37 °C ($P < 0.05$). Nitric oxide levels at 37 °C were higher than at 25–33 °C ($P < 0.05$), and at 35 °C were higher than at 25–31 °C ($P < 0.05$). Blood cell viability at 25–31 °C was higher than at 37 °C ($P < 0.05$). These results suggest that at an environmental temperature of 33 °C, the frogs' body temperature approached 31 °C or higher, and were likely to be harmful to the frogs. Finally, the environmental temperature that caused frog blood cell death was 37 °C.

1. Introduction

Anurans are the amphibians known simply as frogs and toads [1]. They have existed on Earth since the Jurassic Period, over 150 million years ago, and have colonized every continent at different times throughout Earth's history [2]. Frogs are among the most endangered groups of wildlife. According to the 2008 IUCN Red List, one-third of the 6000 amphibian species are at risk of extinction [3]. Over 50 frog species are caught globally for human consumption [1,4].

Frog species that have economic significance are collected both through hunting (Indonesia, Malaysia, Albania, Turkey, India, and

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China) and through farming in some countries (Brazil, Mexico, Uruguay, Ecuador, Thailand, Taiwan, and Vietnam) to be exported to European countries [4]. In certain parts of Asia (such as Indonesia) and Africa (such as Nigeria), frogs are often called "jumping chickens" due to their taste, which is considered similar to chicken. Frog meat is also valued for its excellent taste and texture, often compared to both fish and chicken [5]. Frog consumption holds popularity in some European countries such as France, Belgium, Italy and Luxembourg [4]. Frogs' legs are imported to the EU at 4070 tonnes annually, correlating roughly to 81–200 million frogs [6].

At present, the frog species that is most utilized for culture is *Rana catesbeiana* (Shaw, 1802), reclassified as *Lithobates catesbeianus* (Shaw, 1802), native of North America, which is popularly known as the American bullfrog [7]. The frog *Rana rugulosa* (Wiegmann, 1834), also called wrinkled frog or the Thai native frog is found across East Asia, ranging from Japan and Korea through northeastern China to the southernmost parts of Russia. The frog *Rana tigerina* (Daudin, 1802) is a frog that is commonly found in Thailand, as is the *Rana rugulosa*. A study by Jiwyam et al. [8] found that these two frog species had a close genetic relationship. In Thailand, frog farming has been attempted for many years with varying degrees of success and the number of frog culture farms expanded rapidly in 1992. After that, frog culture has been practiced widely throughout South-east Asia [9]. Three species of frogs have been crossed, namely *Lithobates catesbeianus* x *Rana tigerina* x *Rana rugulosa*, which were developed to be suitable for raising in Thailand [10].

Global warming influences the behavior, distribution, and physiology of numerous animal species. Since pre-industrial times, the global average temperature has risen by 1.0 °C. In the past decade, there have been record-breaking storms, forest fires, droughts, heat waves, and floods worldwide. It is predicted that such extreme weather events and temperature peaks will become more frequent in the future [11]. Climate change is a phenomenon of long-term changes in the distribution of weather patterns [12]. The rise in temperature stands as a paramount indicator of climate change, and consequently leads to the occurrence of various extreme phenomena such as heat waves [13]. Climate change is one of the greatest problems threatening biodiversity and ecosystems in the 21st century, leading to the extinction of many species [14].

Generally, increasing environmental temperature causes aquatic animals to be under heat stress. This condition leads to increased release of the stress hormone, metabolic responses, changes in haemocytes, and alterations of immunity. These responses to heat stress result in changing behaviour, reduced growth and development, reproductive system failure, increased susceptibility to diseases, and in cases of illness, it can be a cause of death [15–17].

Amphibians are one of the most sensitive animal groups to changes in climatic conditions [13,14,18,19]. Like all amphibians, frogs are ectotherms, meaning that their body temperature changes with the temperature of the environment [20,21]. At the physiological level, body temperature of ectothermic animals are found to be highly correlated with environmental temperature. They regulate body temperature through behaviour by selecting suitable microclimates [22]. Rocha and Branco [23] reported that when environmental temperature increased, breathing frequency, body temperature and heart rate were affected [11].

Reactive oxygen species, including the superoxide anion (O2•-), hydrogen peroxide (H_2O_2), and the hydroxyl radical (•OH), are primarily produced within mitochondria under normal physiological conditions. Generally, levels of reactive oxygen species remain stable, but if there is a transient increase, and they are not promptly removed, heightened reactive oxygen species can lead to oxidative stress [24,25]. When frogs are exposed to high environmental temperature, they experience oxidative stress and their total antioxidant capacity decreases [26]. Numerous studies have found that oxidative stress promotes cell death [27]. Nitric oxide is a molecule capable of freely traversing cell membranes. It is a free radical which can be highly reactive. The role of nitric oxide in cellular processes is mainly concentration dependent and nitric oxide has multiple roles involved in physiological function and pathology. In addition, nitric oxide must be present at the appropriate concentration and timing to exert its biological functions [28].

The red blood cells of fish, amphibians, reptiles, and birds possess a nucleus and functional mitochondria, which serve as sources of free radicals [29–31]. Studies by Srinontong et al. [32] and Wandee et al. [33] revealed that exposure of broiler blood cells to high temperatures resulted in increased levels of hydrogen peroxide, nitric oxide, and malondialdehyde, while reducing cell viability.

Ruthsatz et al. [34] who studied the optimum temperature for European common frogs (*Rana temporaria*), found that temperatures between 14 C and 28 °C were the lowest and highest that frogs could tolerate, respectively, and 25 °C was the most suitable temperature for this type of frog. In addition, frogs were able to adapt when temperatures increased about 2–3 °C and no more than 5 °C from a comfortable temperature [35]. Also, Raske et al. [36] found that the optimum temperature for American bull frogs was 24.5–25.4 °C.

Tammadid et al. [37] reported that the average annual temperature of Thailand was 26.8 °C, with the maximum temperature in April being average 32.6 °C and the minimum in January was average at 22.2 °C. In general, although the average temperature in Thailand is not much different from the suitable environmental temperature for frogs, during the summer season, the average environmental temperature is 7 °C higher than the frog's normal comfortable temperature. Amnuaylojaroen et al. [38] reported that the temperature in Thailand would likely increase by 0.62 °C per decade in the future. Therefore, the upward trend in may affect the raising frogs for future consumption and trade.

However, there is limited understanding regarding the impact of environmental temperature on cellular activity in relation to physiological changes in frogs. Therefore, to proactively address potential future challenges, this study aimed to explore how increasing environmental temperature affects physiological changes, oxidative stress, nitric oxide production, total antioxidant capacity, and blood cell viability in American bullfrog crossbreeds. The findings from this investigation will offer crucial foundational insights for future applications in enhancing environmental management strategies for frog farming or conservation efforts.

2. Materials and methods

2.1. Animals

The experiment took place from September to October 2023 at the Faculty of Veterinary Sciences, Mahasarakham University, Mahasarakham, Thailand. It adhered to the Guide for the Care and Use of Laboratory Animals (8th edition, 2011). Ten frogs, crossbred (*Lithobates catesbeianus* x *Rana tigerina* Daudin x *Rana rugulosa* Weigmann), with an average weight of 200 g, were sourced from a local frog farm in Maha Sarakham province. They were housed in a shaded concrete pond measuring 2x4 m. For transportation, the frogs were placed in a 30x40 × 20 cm plastic box with water and a ventilation grate on top. The temperature in the transport vehicle was maintained at 25 °C during the 30-min trip to the university. Upon arrival, the frogs were health-checked and screened for parasites before being placed in glass terrariums (30x50 × 40 cm) with ventilated lids. Each terrarium housed two frogs and included both water and dry areas. The terrariums were kept at room temperature (25 °C) with a 12:12 h light-dark cycle. The frogs were fed commercial feed (30 % CP, 4 % fat, 6 % fiber, 12 % moisture, HI, FROG 3S®, Thailand) once daily in the afternoon from 4:00–4:30 p.m. The feed amount was calculated to be no more than 5 % [39] of their body weight, and any leftover food was removed after 30 min. The frogs were conditioned for 14 days before beginning the experiment.

2.2. Experimental design

This research comprised two sub-experiments:

Sub-experiment 1: Investigating the impact of increased environmental temperature on physiological changes in frogs. Five frogs from different terrariums were randomly selected and placed in 20 × 30 cm nylon bags to prevent them from jumping. Prior to the experiment, these frogs were acclimatized in a 30x50 × 25 cm box with water maintained at 25 °C. The water temperature was then gradually increased by adding warm water, monitored with a digital thermometer, to 25, 27, 29, 31, and 33 °C. Frogs were placed in water at each specified temperature for 5 min, after which their body temperature, pulse rate, ventilation rate, and blood oxygen saturation were measured.

Sub-experiment 2: Assessing the effect of increased environmental temperature on total antioxidant power, hydrogen peroxide, malondialdehyde, nitric oxide, and the viability of frog blood cells. Blood samples (2.0 mL) were taken from two frogs using a 3 mL syringe and a 21G sterile needle, collected from the heart [40]. The blood from the two frogs was combined and mixed thoroughly, then diluted with PBS at pH 7.4. This diluted blood was centrifuged at 2500 rpm (769×*g*) for 5 min at 25 °C, with the supernatant discarded after each of two washes. The blood was further diluted at a 1:200 (v/v) ratio with PBS pH 7.4, and 10 mL aliquots were placed in test tubes. The water temperature in a 30x50 × 25 cm box was set to 25, 27, 29, 31, 33, 35, and 37 °C. The blood samples (five replicates per temperature) were maintained at these temperatures for 20 min before being returned to room temperature (25 °C). The supernatant and blood cells in the pellet were then analyzed for total antioxidant power, malondialdehyde, nitric oxide, hydrogen peroxide, and blood cell viability.

2.3. Determination of indicators

1. Physiological parameters

Oxygen saturation and pulse rates in the frog were recorded with a Veterinary Pulse Oximeter (VE-H100B) by attaching the device to the toe membrane. Body temperature was taken using a JENWAY 3345 Ion Meter, by inserting the probe 1 cm into the cloaca for 1 min. Ventilation rate was determined by counting buccal oscillations, the movements of the buccal cavity floor [41], for 1 min.

2. Biochemical parameters

2.1 Total antioxidant power

The total antioxidant power was assessed using the Ferric Reducing Antioxidant Power (FRAP) assay. To prepare the FRAP reagent, 10 mL of 300 mmol acetate buffer (pH 3.6) was combined with 1 mL of 10 mmol 2,4,6-Tris(2-pyridyl)-*s*-triazine and 1 mL of 20 mmol $FeCl_3.6H_2O$. Subsequently, 20 μl of the supernatant was added to 180 μl of the FRAP reagent and the mixture was incubated at room temperature for 5 min. The absorbance was measured at 595 nm, with $FeSO_4.7H_2O$ serving as the standard [32].

2.2 Malondialdehyde

The process for determining malondialdehyde levels was conducted as follows: A mixture of 0.01 mL of the supernatant, 3 mL of 0.05 mol/L HCl, and 1 mL of 0.67 % C4H4N2O2S was prepared. This mixture was then heated at 100 °C for 30 min, allowed to cool, and supplemented with 4 mL of $C_4H_{10}O$. Following vortexing and subsequent centrifugation at 1008×*g* for 10 min, the absorbance was measured at 450 nm. The standard utilized for this measurement was C7H16O4 [42].

3. Nitric oxide

For the determination of nitric oxide levels in the supernatant, the following procedure was employed: Griess's reagent was

prepared by combining $C_6H_8N_2O_2S$ (1 % w/v), $C_{12}H_{16}C_{l2}N_2$ (0.1 % w/v), and H3PO4 (1.25 % v/v) in a bottle, followed by the addition of distilled water to attain a final volume of 100 mL, which was then thoroughly mixed. Equal volumes of Griess's reagent and the supernatant were mixed together and allowed to incubate for 15 min. Subsequently, the absorbance was measured at a wavelength of 540 nm, with NaNO2 utilized as the standard reference [43].

4. Hydrogen peroxide

In the hydrogen peroxide measurement process, 1 mL of 2.25 mmol $FeSO_4.7H_2O$ was initially added to a test tube. Subsequently, 1 mL of the supernatant was introduced and allowed to undergo a 5-min reaction period. Then, 1 mL of 4 mmol $C_{16}H_{18}FN_3O_3$ was added, followed by a 3-min reaction time. The absorbance was then measured at 440 nm, utilizing H_2O_2 as the standard [44].

5. Viability

The survival rate of frog blood cells was assessed using the following procedure: $C_{18}H_{17}N_5S$ was dissolved in CH_3COCH_3 to a concentration of 5 mg/mL and subsequently filtered. 1 mL of the diluted blood sample was placed in a microtube and centrifuged at 769×*g* for 5 min, with 900 μl of the supernatant then being removed. The remaining blood was transferred into a well plate, where 10 μl of $C_{18}H_{17}N_5S$ was added, mixed, and incubated at 41.5 °C for 75 min. Following this, 95 μl of the supernatant was discarded, and 150 μl of $(CH_3)_2SO$ was added. The absorbance was measured at 540 nm [45]. The cell viability percentage was calculated using the formula: % cell viability = (optical density, OD of the treatment groups/OD of the control group) × 100.

2.4. Statistical analysis

The normal distribution of data was tested. Analysis was performed using PROC GLM. Means were separated using Duncan's Multiple Range Tests (SAS® studio). A significance level of $P < 0.05$ was used to determine significant differences.

3. Results

As shown in Fig. 1, when frogs were incubated at temperatures from 25 °C to 33 °C, their body temperature increased continuously for every 2 °C increase ($P < 0.05$) and was highest at 33 °C.

At 33 °C, the frog's pulse rate was significantly higher than at temperatures between 25 °C and 31 °C ($P < 0.05$). Between 27 °C and 29 °C, the frog's pulse rate was significantly higher than that at 25 °C and 31 °C ($P < 0.05$). However, there was no significant difference in pulse rate between 27 °C and 29 °C ($P > 0.05$). Additionally, the frog's pulse rates from 25 °C to 31 °C did not show significant differences ($P > 0.05$) (Fig. 2).

The frog's ventilation rate at 31 °C was significantly higher than at both 25 °C and 28 °C ($P < 0.05$). However, there were no significant differences in ventilation rate between 29 °C and 33 °C ($P > 0.05$). Similarly, the ventilation rates at 25 °C and 28 °C did not differ significantly ($P > 0.05$) (Fig. 3).

There were no significant differences in the frog's oxygen saturation across the temperature range of 25–33 °C ($P > 0.05$) (Fig. 4).

The total antioxidant capacity of the frog's blood cells at 25 °C was significantly greater than at temperatures ranging from 27 °C to 37 °C ($P < 0.05$). At 31 °C, the antioxidant capacity was significantly higher compared to 37 °C ($P < 0.05$). However, there were no significant differences in antioxidant capacity at 27 °C, 29 °C, and between 33 °C and 37 °C ($P > 0.05$) (Fig. 5).

The level of hydrogen peroxide in the frog's blood cells was significantly higher at 27 °C compared to 25 °C and the range of 31–37 °C ($P < 0.05$). However, the hydrogen peroxide level at 27 °C was not significantly different than at 29 °C ($P > 0.05$). Additionally, the hydrogen peroxide levels at 25 °C and 31–37 °C showed no significant differences ($P > 0.05$) (Fig. 6).

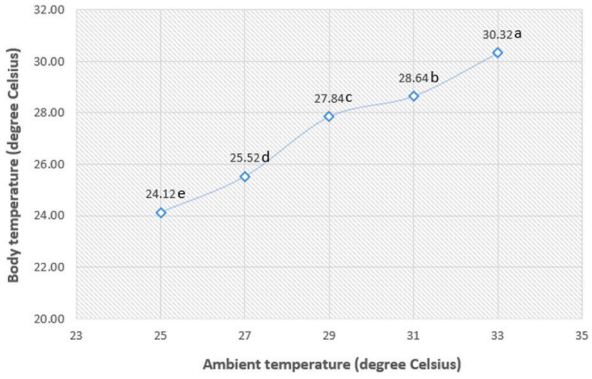

Fig. 1. The body temperature of frogs in an environmental temperature range from 25 to 33 °C; a, b, c, d and e indicate means with statistically significant differences ($P < 0.05$); *P*-value <0.0001; Standard error of the mean (SEM) = 0.33.

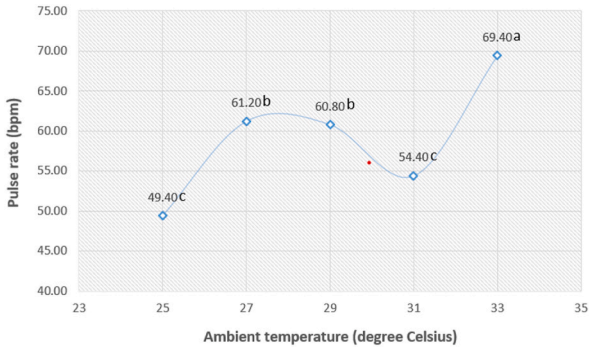

Fig. 2. The pulse rate of frogs in an environmental temperature range from 25 to 33 °C; a, b and c indicate means with statistically significant differences ($P < 0.05$); *P*-value <0.0001; Standard error of the mean (SEM) = 0.97; bpm = Breaths per minute.

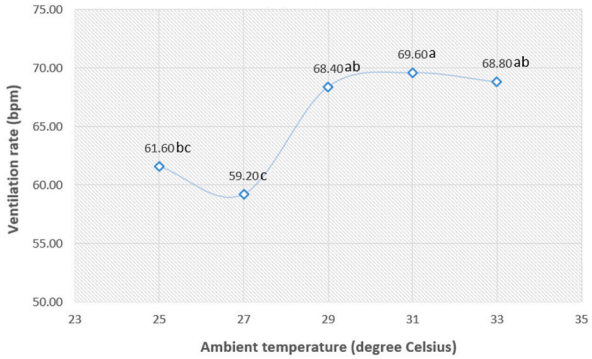

Fig. 3. The ventilation rate of frogs in an environmental temperature range from 25 to 33 °C; a, b and c indicate means with statistically significant differences ($P < 0.05$); *P*-value = 0.0192; Standard error of the mean (SEM) = 1.05; bpm = Breaths per minute.

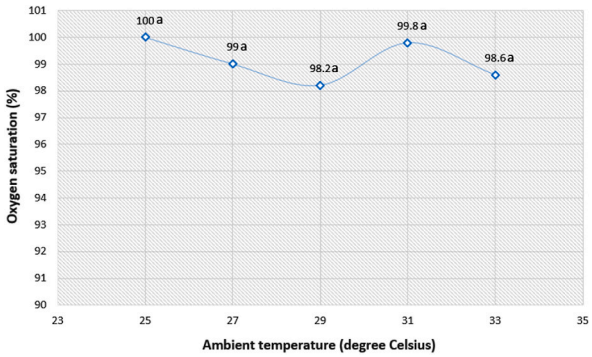

Fig. 4. The oxygen saturation of frogs in an environmental temperature range from 25 to 33 °C. Means with the same letter are not statistically significant differences ($P < 0.05$). *P*-value = 0.2754; Standard error of the mean (SEM) = 0.54; % = Percentage.

The malondialdehyde levels in the frog's blood cells at temperatures between 25 °C and 33 °C were significantly higher than at 35 °C and 37 °C ($P < 0.05$) (Fig. 7).

The nitric oxide levels in the frog's blood cells were significantly higher at 37 °C compared to temperatures ranging from 25 °C to 33 °C ($P < 0.05$). At 35 °C, the nitric oxide levels were also significantly higher than at temperatures between 25 °C and 31 °C ($P < 0.05$). However, there were no significant differences in nitric oxide levels among temperatures ranging from 25 °C to 33 °C ($P > 0.05$) (Fig. 8).

The viability of the frog's blood cells ranging from 25 °C to 31 °C was significantly higher than at 37 °C ($P < 0.05$). However, there were no significant differences in viability between temperatures from 25 °C to 35 °C ($P > 0.05$) (Fig. 9) (see Fig. 10).

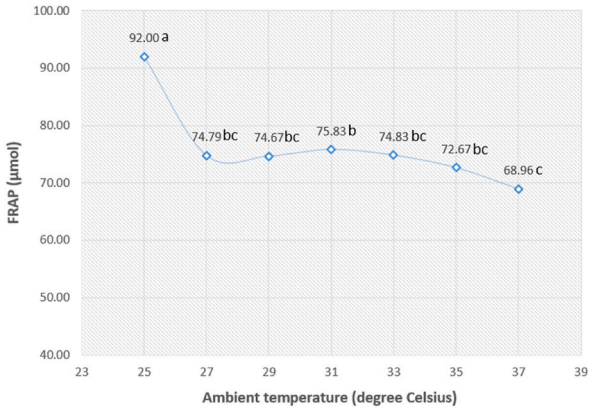

Fig. 5. The total antioxidant power (FRAP) of frog blood cells in an environmental temperature range from 25 to 37 °C; a, b, and c indicate means with statistically significant differences ($P < 0.05$); *P*-value <0.0001; Standard error of the mean (SEM) = 0.99; µmol = Micromolar.

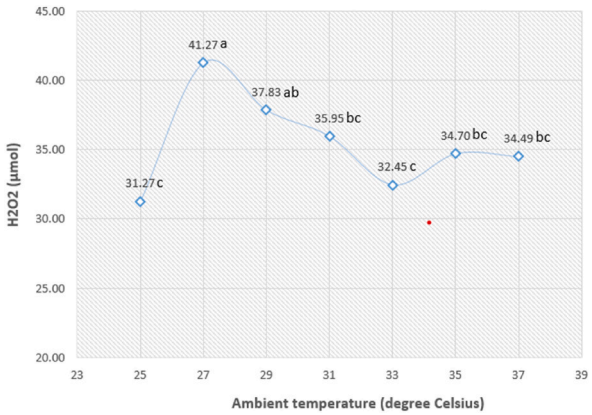

Fig. 6. The hydrogen peroxide (H_2O_2) of frog blood cells in an environmental temperature range from 25 to 37 °C; a, b, and c indicate means with statistically significant differences ($P < 0.05$); *P*-value = 0.0043; Standard error of the mean (SEM) = 0.91; µmol = Micromolar.

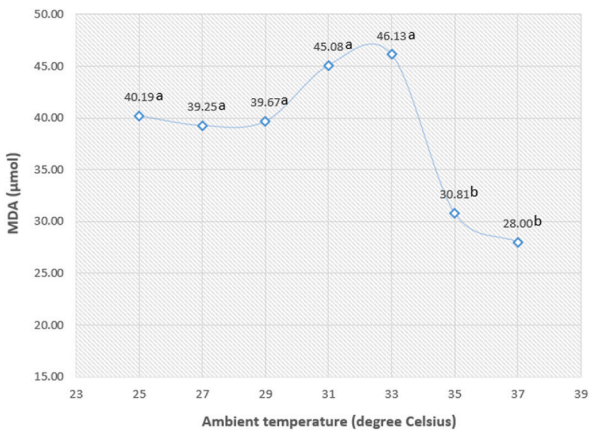

Fig. 7. The malondialdehyde (MDA) of frog blood cells in an environmental temperature range from 25 to 37 °C; a, b, and c indicate means with statistically significant differences ($P < 0.05$); *P*-value = 0.0002; Standard error of the mean (SEM) = 1.14; µmol = Micromolar.

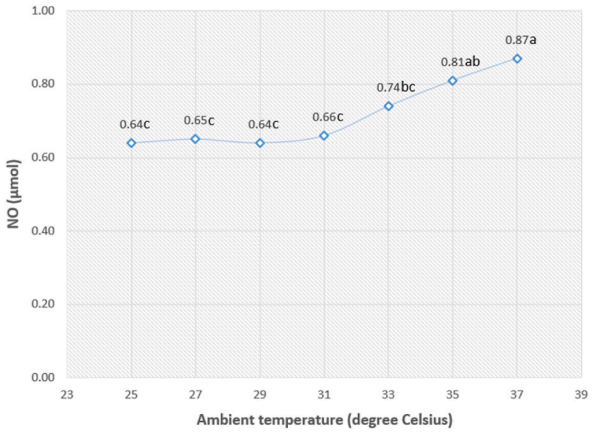

Fig. 8. The nitric oxide (NO) of frog blood cells in an environmental temperature range from 25 to 37 °C; a, b, and c indicate means with statistically significant differences ($P < 0.05$); *P*-value = 0.0001; Standard error of the mean (SEM) = 0.14; µmol = Micromolar.

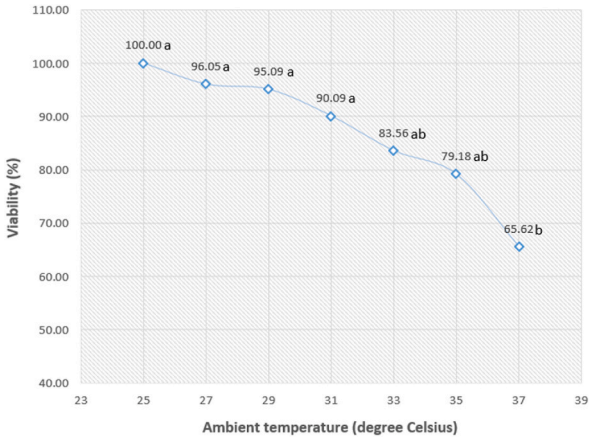

Fig. 9. The viability of frog blood cells in an environmental temperature range from 25 to 37 °C; a and b indicate means with statistically significant differences ($P < 0.05$); *P*-value = 0.0224; Standard error of the mean (SEM) = 2.04; % = Percentage.

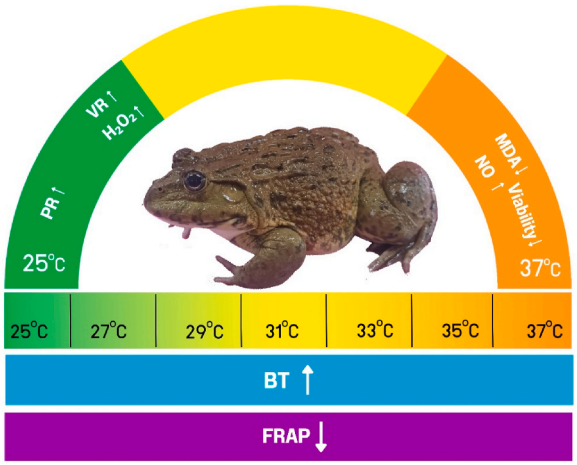

Fig. 10. Summary of the overall effects of increased environmental temperature on the physiological and biochemical changes in American bullfrog crossbreeds (BT = Body temperature; FRAP = Total antioxidant power; PR = Pulse rate; VR = Ventilation rate; H2O2 = Hydrogen peroxide; MDA = Malondialdehyde; NO = Nitric oxide; Viability = Blood cell viability).

4. Discussion

Amphibians have complex life cycle dependencies because they live on both land and water [11]. It has been found that environmental temperature is an important factor for these animals. Therefore, knowing their ability to withstand environmental temperatures which correlate to physiological and biochemical changes will be useful to manage both husbandry and conservation of these amphibians in the advent of future global warming crises [46].

Temperature is a key environmental factor that affects amphibians, such as frogs [47], because as temperature rises, the rates of most biochemical reactions and numerous biological processes tend to accelerate [34] and temperature influences physiological changes [48]. Generally, the temperature of amphibians depends on the temperature of the environment, therefore, the body temperature of these animals is strongly influenced by the surrounding environmental temperature [49]. In this study, the body temperatures of the frogs rose in response to elevated environmental temperatures, increasing from 25 °C to 33 °C in 2 °C increments, reaching its peak at 33 °C. This finding is in line with the reports of Vences et al. [48], Navas et al. [50], Gómez-Hoyos et al. [49] and Ruthsatz et al. [47] who studied the impact of environmental temperature on frogs discovered that increasing the surrounding temperature resulted in a corresponding rise in the frogs' body temperature.

It is well-established that in ectothermic animals, heart rate rises as temperature increases [51]. The endocrine and autonomic nervous systems [52], including the direct effect of temperature on pacemaker cells [51], play crucial roles in adequate oxygen delivery to metabolically active tissues. As metabolic demands shift, adjustments in cardioventilation are typically necessary [11]. In this study, when environmental temperature increased, the pulse rate of the frog at 33 °C was higher than between 25 and 31 °C. This occurrence indicated that high environmental temperature caused increased pulse rate. This phenomenon is in accordance with the studies of Barcroft and Izquierdo [53], Rocha and Branco [23], Rocha and Branco [52] who found that when the environmental temperature rose, heart rate of frogs increased. Also, the pulse rate of the frogs at 27 °C and 29 °C was higher than at 25 °C, but at 31 °C, the pulse rate decreased below 27 °C and 29 °C, even if the body temperature of the frogs still continuously increased. At this temperature (31 °C), the ventilation rate of the frogs was also higher than at 25 °C and 27 °C. This result is consistent with the report by Trachsel et al. [54], which found that short-term acclimation to hyperthermia led to limited adaptations in cardiac function during heating. The extent of these adaptations depends on various factors, such as the duration of exposure, frequency, total number of exposures, and the environmental conditions in which acclimation occurs [55]. This result was different from that of Smith [56] who found that the heart rate curve was characterized by a continuous increase when the environmental temperature was raised. However, his study was conducted at a maximum temperature of only 21 °C, but in the present study, we found a decrease in pulse rate at 31 °C.

In this study, the ventilation rate of the frog was higher at 31 °C compared to 25 °C and 29 °C. This result corresponded with the reports of Rocha and Branco [23] and Rocha and Branco [52] who found that as the environmental temperature rose, the ventilation rate of the winter bullfrog *Rana catesbeiana* and the bullfrog *Rana Catebein* also increased. Whitford [57] and da Silva et al. [58] explained this phenomenon as follows; when frogs are exposed to high environmental temperatures, their pulmonary ventilation adjusts to balance oxygen demands with carbon dioxide elimination. Higher temperatures lead to increased oxygen consumption and reduced oxygen affinity of hemoglobin. Additionally, the combination of elevated temperatures and hypoxia results in a significant increase in ventilation, which can be attributed to the balance between higher oxygen demand, lower oxygen supply, and increased sensitivity of oxygen chemoreceptors [58,59].

Arterial blood oxygen saturation is the most commonly used parameter for evaluating pulmonary gas exchange [60]. The primary reason for measuring this parameter is to detect hypoxia [61]. Generally, in humans, when hypoxia occurs, the level of SpO_2 is less than 95 % [62]. In the present study, it was found that the oxygen saturation of the frog between 25 and 33 °C did not vary. This phenomenon showed that when frogs are exposed to high environmental temperatures, they still have enough oxygen supply for living; this may be due to the increase in the respiratory and cardiovascular systems function and these systems are essential for maintaining sufficient oxygen levels [62].

The antioxidant system generally consists of enzymatic antioxidants, such as catalase, glutathione peroxidase, glutathione reductase, and superoxide dismutase, as well as non-enzymatic antioxidants, such as glutathione etc. [26]. The measure of total antioxidant power can be determined using all the antioxidants which were present in samples [63]. This method is one of the most widely used [64]. In this experiment, the total antioxidant power of frog blood cells was higher at 25 °C compared to 27–37 °C, and it was higher at 31 °C compared to 37 °C. This result showed that when frog blood cells are exposed to high environmental temperatures, their total antioxidant power decreased. However, this was different from the report of Zhang et al. [25] who found that when the frog *Nanorana parkeri* was exposed to acute warming, the total antioxidant capacity increased. Also, antioxidant enzyme activity, i.e., glutathione peroxidase, catalase, and glutathione-S-transferase also increased in their tissue. However, the experiment of Zhang et al. [25] studied increasing environmental temperature from 4 °C to 10 °C, which is clearly different from this study where the temperature range was 25–37 °C. It is possible that during low environmental temperatures, an acute increase in temperature will enhance the frog's antioxidant capacity. On the other hand, high environmental temperatures reduce the total antioxidant power.

The generation of hydrogen peroxide in cells involves the breakdown of the superoxide anion that is generated by reduction of oxygen during aerobic respiration and its rapid transformation by superoxide dismutase [65]. Hydrogen peroxide can produce hydroxyl radicals through a Fenton reaction, and these free radicals cause damage to cellular proteins, lipids, and nucleic acids, ultimately leading to apoptosis. Additionally, exposure to hydrogen peroxide induces apoptosis via the mitochondrial pathway [66]. In this study, the hydrogen peroxide levels in frog blood cells were higher at 27 °C than at 25 °C and 31–37 °C. This phenomenon indicated that at 27 °C, frog body cells produced higher levels of hydrogen peroxide than at other environmental temperatures. The results of this study are consistent with the findings of He et al. [26], who reported that exposure to high temperature environments and heat waves increased hydrogen peroxide levels in both the liver and muscle of *Nanorana pleskei* frogs.

Malondialdehyde is a compound derived from the peroxidation of polyunsaturated fatty acids. It serves as a biomarker for measuring oxidative stress in various biological samples [67]. In this study, the malondialdehyde of the frog blood cells between 25 and 33 °C was higher than at 35 °C and 37 °C. The result showed that at environmental temperatures between 25 and 33 °C, frogs were not under oxidative stress. In addition, at 35 °C and 37 °C, malondialdehyde also decreased. This observation aligns with the findings of Zhang et al. [25], who observed a decrease in malondialdehyde levels in brain and liver tissue when *Nanorana parkeri* frogs were exposed to acute heat. The malondialdehyde of frogs at temperatures ranging between 25 and 33 °C did not change with increasing temperature. This was associated with a decrease in total antioxidant power at temperatures between 27 and 37 °C. It is possible that malondialdehyde did not change at 25–33 °C because the frog's antioxidant system has a high capacity to prevent the occurrence of oxidative stress when subjected to high environmental temperatures.

Nitric oxide (NO) serves as a pivotal signaling molecule in nearly all organisms, playing a central role in signaling pathways associated with various physiological and pathological processes [68]. Another function of nitric oxide is that it acts as an antioxidant, especially to lipid peroxidation. In addition, nitric oxide can also scavenge reactive oxygen species directly [28]. In this experiment, the level of nitric oxide in frog blood cells was observed to be higher at 37 °C compared to temperatures ranging from 25 °C to 33 °C. Moreover, at 35 °C, there was a significant increase in nitric oxide levels compared to the range of 25–31 °C. These findings suggested that as the environmental temperature rose to 35 °C and 37 °C, there was an elevation in nitric oxide levels within frog blood cells, which correlated with a decrease in malondialdehyde, a product of lipid peroxidation.

Excessive heat accumulation due to elevated environmental temperature poses a risk for cell death. Mitochondria function both as a source of oxygen radicals and as sensors for oxidative stress. Furthermore, they play a crucial role in regulating apoptosis in response to various signals inducing cell death, such as heat stress [69]. This study found that frog blood cell viability was higher at temperatures between 25 °C and 31 °C compared to 37 °C. This finding aligns with the reports by Zhao et al. [70] and Kayastha et al. [71] who studied the effects of heat stress on cell death of largemouth bass *Micropterus salmoides* and *Paramacrobiotus experimentalis* Kaczmarek, Mioduchowska, Poprawa, & Roszkowska, 2020, respectively. However, when considering the level of malondialdehyde as an indicator of oxidative stress, it was found that at 35 °C and 37 °C, the malondialdehyde level decreased, and the viability of the frog's blood cells also decreased. Therefore, the decrease of cell viability may not have been caused by oxidative stress, but by high temperature that directly stimulates cell death.

The primary conclusion from this study is that as the environmental temperature rose from 25 °C to 33 °C, the body temperature of frogs increased correspondingly, yet remained consistently lower than the environmental temperature. At an environmental temperature of 25 °C, the frogs' body temperature was approximately 0.88 °C lower. As the environmental temperature increased, the gap between the frogs' body temperature and the environmental temperature widened. Specifically, at an environmental temperature of 33 °C, the frogs' body temperature was found to be 30.32 °C, showing a difference of approximately 2.68 °C from the environmental temperature.

For blood cells, it was observed that at 31 °C, the levels of nitric oxide and the viability of frog blood cells did not differ from those at 25 °C. These parameters began increasing and then decreasing at 33 °C and 35 °C, respectively. At higher temperatures, nitric oxide and viability of frog blood cells increased and decreased statistically up to 37 °C. Therefore, it can concluded that the frog's body cells can tolerate a maximum environmental temperature of 31 °C because the viability of the frog blood cells was not different from at 25 °C. Because the corresponding change between physiology and body cell of the frog was found at 31 °C. It was found that at this temperature the ventilation rate of frogs increased from that at 27 °C, which is a physiological response to bring oxygen into the body. On the other hand, the pulse rate of the frog at 31 °C, dropped below that at 27 °C and 29 °C, and then increased again at 33 °C, which was higher than at temperatures between 25 and 29 °C. It was found that hydrogen peroxide in frog blood cells increased between 27 and 29 °C and then decreased at higher temperatures until it was no different from that at 25 °C, while total antioxidant power dropped between 27 and 37 °C. This finding is consistent with malondialdehyde not changing between 25 and 33 °C and may be due to the frog's antioxidants being used for neutralizing free radicals, resulting in a decrease in total antioxidant power. However, in the temperature range between 35 and 37 °C, total antioxidant power and malondialdehyde of frog blood cells decreased. This finding may have been caused by increased levels of nitric oxide, which reduces lipid peroxidation, resulting in a decrease in malondialdehyde. The decrease in total antioxidant power might be caused by a reduction in the viability of frog blood cells, resulting in a subsequent decrease in the total antioxidant power from the frog's blood cells.

5. Conclusion

Live frogs and frog blood cells were studied in environments subject to 2 °C incremental steps in the range 25–33 °C and 25–37 °C, respectively. Physiological parameters of the live frogs, and biochemical parameters and viability of frog blood cells were measured. It was found that the body temperature of frogs increased continuously as the environmental temperature rose, but the body temperature of frogs did not increase as much as the environmental temperature. In addition, frogs could withstand environmental temperatures up to a maximum aT 33 °C. At such a temperature, frogs had an average body temperature of 30.32 °C. When compared to frog blood cells at 31 °C, various parameters including hydrogen peroxide, malondialdehyde, nitric oxide and the viability of frog blood cells are not different from those at 25 °C except for total antioxidant power, which decreased due to an increase in free radicals at 27 °C. At temperatures of 35–37 °C, it was found that malondialdehyde and total antioxidant power decreased, possibly due to an increase in nitric oxide and a decrease in the viability of frog blood cells, respectively. The results of this study revealed that environmental temperatures of 33 °C and above or frog body temperatures of 31 °C and above are likely to be harmful to frogs. Therefore, if the environmental temperature increases to that temperature (environmental temperatures of 33 °C and above or frog body temperatures of 31 °C), and without proper environmental management, it may cause frog death due to heat stress.

6. Future research

This study investigated physiology and biochemistry information related to the adaptation of live frogs and body cells of frogs to high temperatures. The results showed that environmental temperatures affect frog survival and blood cell viability. Therefore, further research should study factors influencing frog adaptation to high-temperature environments and potential solutions.

7. Limitations of the study

Prior to initiating the experiment, a preliminary study was performed to identify an appropriate temperature range that would avoid causing severe stress or mortality in the frogs. The temperature range selected was between 25 and 33 °C. Temperatures higher than this caused severe heat stress. In order to comprehend the physiological and biochemical alterations in frogs upon exposure to elevated environmental temperatures, we used frog blood cell *in vitro* and raised the environmental temperature to 37 °C, at which cell viability decreased. However, in reality, cell death began to occur at 33 °C and 35 °C, but the difference became very clear at 37 °C.

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Data availability

All data relevant to this study has included in article. For any additional data requirements beyond what is presented, we are more than willing to provide it upon request.

Ethics statement

This study was approved by the Institution's Ethics Committee on Animal Experimentation of Mahasarakham University (license number: IACUC-MSU-46/2023).

CRediT authorship contribution statement

Rattanatrai Chaiyasing: Writing – review & editing, Methodology, Investigation, Conceptualization. **Pailin Jinagool:** Writing – review & editing, Investigation. **Vajara Wipassa:** Writing – review & editing, Investigation. **Prayuth Kusolrat:** Writing – review & editing, Investigation. **Worapol Aengwanich:** Writing – review & editing, Writing – original draft, Validation, Supervision, Software, Methodology, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

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

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