



Research article

Hoven's carp *Leptobarbus hoevenii* strategized metabolism needs to cope with changing environment

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ABSTRACT

Current water warming and freshwater acidification undoubtedly affect the life of aquatic animals especially ammonotelic teleost by altering their physiological responses. The effect of temperature (28 °C vs 32 °C) and pH (7 vs. 5) on the metabolic compromising strategies of Hoven's carp (*Leptobarbus hoevenii*) was investigated in this study. Fishes were conditioned to (i) 28 °C + pH 7 (N_{28°C}); (ii) 32 °C + pH 7 (N_{32°C}); (iii) 28 °C + pH 5 (L_{28°C}) and (iv) 32 °C + pH 5 (L_{32°C}) for 20 days followed by osmorespiration assay. Results showed that feeding performance of Hoven's carp was significantly depressed when exposed to low pH conditions (L_{28°C} and L_{32°C}). However, by exposed Hoven's carp to L_{32°C} induced high metabolic oxygen intake and ammonia excretion to about 2x-folds higher compared to the control group. As for energy mobilization, Hoven's carp mobilized liver and muscle protein under L_{28°C} condition. Whereas under high temperature in both pH, Hoven's carp had the tendency to reserve energy in both of liver and muscle. The findings of this study revealed that Hoven's carp is sensitive to lower water pH and high temperature, thereby they remodeled their physiological needs to cope with the environmental changes condition.

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1. Introduction

Freshwater warming trend was anticipated to accelerate worldwide as it has directly associated with the ongoing global warming scenario in response to increase in CO₂ [1,2,3]. The global land and ocean temperature in two decades were recorded at 0.84–1.10 °C higher than in 1850–1900 and the global temperature is expected to exceed 1.5 °C if CO₂ emissions keep rising [4,5]. Carbon originated from nature events and nevertheless, the impact of nature releasing CO₂ to the environment from the decomposition process [6,7], respiration [8] and volcano activity [9] are not critical as compared to anthropogenic activity.

The phenomenon occurrence of ocean acidification is well documented due to excessive amount of CO₂ absorption generated by human activities [10,11,12] and its impacts on the whole ecosystem have been studied intensively for many taxa [13,14]. Ocean surface is known to be proportionated to the atmosphere to absorb CO₂ that resulted in acidifying ocean as consequence [15]. Acidification is more complex in freshwater [16,12] where the major contributions were from sulphuric and nitric acids [17,18] that were associated with fossil fuel uses [14,19,20], mining [21,22], urbanization [23], water waste discharges [24], run-off from agriculture and industrial area and decomposition of organic and inorganic carbon [18,25]. These activities and waste production sink into the freshwater body via precipitation volume [26], which ends up directly into the freshwater basin or through rainfall-runoff [27,28]. In worst cases, freshwater acidification can be long-lasting, and the recovery takes decades at the expanses of time and cost [29,30]. In addition, as plants grow, they absorb large amount of CO₂ via the photosynthesis process, and when the plants die, the breakdown release organic carbon into the system [12,31].

The severity of ongoing warming and acidification alters the freshwater ecosystem and its biota [32,12]. Warming temperature was more pronounced in tropical species due to their lower adaptive capacity toward temperature fluctuation in comparison with temperate species that were more resilient to seasonal temperature variation [33,34,35]. The warming is known to affect all levels of organisms from the smallest planktons to mollusks, aquatic insects [36] and up to the largest teleost [37,38]. Physiological responses to environmental changes are relatively important as they reflect the well-being of the animal in regulating homeostasis balance toward the external environment. Well, adapting to changing environment can be classified into three categories which are primary (endocrine changes), secondary (physiological changes and adjustment related to metabolism, respiration, hydromineral balance, cardiovascular, immune function, and cellular response) and tertiary (overall performance such as growth, disease resistance and behavior) [39,40].

As a thermoregulatory animal, each species has a varied thermal tolerance range with biochemical reactions related to temperature-dependent for effective function [41,42,43,44,45]. An increase in temperature above their optimum physiology threshold will induce an increase in oxygen demand to cope with an increase in metabolic rate [46]. The situation may worsen if food availability became scarce as energy will be reallocated for maintenance, voluntary physical activity, and heat increment (digestion, absorption, and excretion) instead of growth and reproductive purposes [47,48]. Subsequently, altering basal metabolism needs with extra energy expenditure required to support all biological processes [49]. Previous attention had been focused on the effects of freshwater acidification on freshwater species in the earlier decade [18] on physiological alteration [50,51], food-web balancing [52], early life stage performances [53], growth reduction [54], reproductive impairment, swimming performance reduction [55], behavioural responses [15], ionic imbalance, energy expenditure alteration [56,57] and foraging efficiency of aquatic animals [58]. Successfulness adaptability to low pH environment is depend on species-specific, life stages [59,60], size [61] and branchial plasticity remodeling [62] as a major role in regulating physiological [63,64,65] and biochemical needs [66] to survive under low water pH.

Hoven's carp belongs to Cyprinidae family and is classified as omnivorous species [67]. Hoven's carp is an endemic freshwater species to Southeast Asia, including Malaysia, Thailand, Indonesia, Laos, Vietnam and Cambodia, commonly found in rivers, streams or seasonal floodplains [68,67]. Female Hoven's carp reached a sexually mature size at a body weight of 350g with a fecundity of about 35000 eggs while male fish matured sexually at a body weight of 180g [69]. As an omnivorous fish, *Moina* and *Daphnia* sp. are the main exogenous diets for Hoven's carp after hatched [70]. At the age of juvenile onwards, Hoven's carp mainly eat plant-based food and small crustaceans in natural water. For aquaculture purposes, commercial feed/pellets have been used for growth production [71]. Hoven's carp not only serve as important freshwater food fish in Asia [72,73,74], but also trade as ornamental fish [73,75]. Nevertheless, most of the literature information available on Hoven's carp were focused on the aspects of breeding techniques [76,77] larvae, development [70], dietary nutrients and growth [78,79], transportation [80], histopathological [62], health [81], sensory modalities [82] and food processing technique [83]. Research about physiological responses towards changing environments is relatively limited. Furthermore, Hoven's carp population in the wild has been reported declining [84] and is listed as moderately threatened under IUCN Red List of threatened species due to habitat disturbance, overfishing and endemism [85]. Thus, this species was chosen as the study model. To the best of our knowledge, interactive impact temperature and pH on physiological plasticity of Hoven's carp such as bioenergy mobilization pattern and osmo-ionoregulation still remain to be evaluated. Therefore, this study was aim to examine the growth and physiological responses of Hoven's carp when exposed to an increase temperature and low water pH on the growth, osmoregulation strategies and energy mobilization pattern.

2. Materials and methods

2.1. Experimental animals and maintenance

A total of 40 Hoven's carp, *L. hoevenii*, average body weight (BW) of 22.46 ± 0.95 g and total length (L) of 11.45 ± 0.17 cm, were purchased from the aquarium shop (KT Aquarium Pet Shop) Kuala Terengganu and were transferred and maintained in the Institute of

Tropical Aquaculture and Fisheries (AKUATROP) hatchery facility, Universiti Malaysia Terengganu (UMT). The samples (40 fish) were randomly distributed equally ($n = 10$) into four holding glass aquariums sized at $1.5 \times 1.5 \times 3.0$ ft supplied with dechlorinated tap water at a temperature of 28.1 ± 0.5 °C and ionic composition of 0.132 Na^+ , 0.069 Cl^- , 0.037 K^+ , 0.043 Ca^+ and 0.003 Mg^{2+} mmol/L. Each aquarium was equipped with an external filter using a bio-mechanical filtration system that contained wadding, bio-balls, and gravel sand to remove particle waste and promote the nitrification process. Water qualities were monitored by using API Freshwater Master Test Kits (MARS®Fishcare Brands, UK) with $\text{NH}_3/\text{NH}_4^+ < 0.25$ mg/L, $\text{NO}_2^- < 0.25$ mg/L and $\text{NO}_3^- < 20$ mg/L. Dissolved oxygen was always maintained at > 6.0 mg/L and monitored by using a Portable Dissolved Oxygen Meter (Waterproof CyberScan DO 300, Eutech Instruments/Oakton Instruments, USA). Water pH was checked by using an Eco pH + meter (Trans Instruments, Singapore) and managed at pH 7. During the acclimatised period, all fish were fed twice a day *ad libitum* for 14 days prior to experimentation.

2.2. Experimental design

The experiment was executed with a 2×2 factorials design with temperatures (28 °C as normal temperature versus 32 °C as high temperature) and water pH (pH 7 as neutral versus pH 5 as acidic) denoted as (a) 28 °C + pH 7 ($\text{N}_{28^\circ\text{C}}$; $n = 10$), (b) 32 °C + pH 7 ($\text{N}_{32^\circ\text{C}}$; $n = 10$), (c) 28 °C + pH 5 ($\text{L}_{28^\circ\text{C}}$; $n = 10$) and (d) 32 °C + pH 5 ($\text{L}_{32^\circ\text{C}}$; $n = 10$). Fish conditioned to $\text{N}_{28^\circ\text{C}}$ were referred to as control. The four glass aquariums ($1.5 \times 1.5 \times 3.0$ ft) were randomly labelled for four treatments and subjected to experimental conditions for 20 days ($n = 10$). The water temperature opted for this experiment was based on the results obtained from preliminary experiments [62], where fish were able to survive and maintain their feeding performance at an optimum level of about 2.8 % BW or 6.40 ± 0.94 g/day under combined effects of high temperature (32.3 ± 0.5 °C). This temperature was also reported as an average annual higher temperature reported by the [86].

To achieve the desired water temperature, the temperature was gradually increased by 0.5 °C daily to ± 32 °C using a regulated aquarium thermostat heater (RS-978, 500W, China). Water pH was adjusted slowly to reach the targeted acidic water pH by using 1 mM hydrochloric acid (HCl). HCl was added slowly into the reservoir to achieve low pH of 5.0 at a rate of about 0.5 units per day controlled by using a peristaltic pump (SOGO, GB37-530). The pH value was monitored and recorded every 2h by using an Eco pH + meter (Trans Instruments, Singapore). Weekly, 50 % of water was renewed with pre-conditioned water from a stock prepared according to experimental conditions. At water temperature and pH achieved desired level, all fishes were maintained under targeted experimental conditions for 20 days.

2.3. Feeding, growth performance and fitness indexes

Ad libitum feeding was performed twice daily at 0830h and 1700h with a commercial feed (TP- 1, STAR FEED) containing nutritional composition of 28 % crude protein, 3 % crude fat and 12 % moisture. The amount of feed consumed were weighed and recorded daily for 20 days of the experimental period to determine the feed intake. Uneaten food after 15 min were removed, dried, and reweighed to determine the total feed intake per meal. Growth performances such as total feed intake ($\text{TF}_{\text{intake}}$, g/fish), relative weight gain (RWG, %/g) and specific growth rate (SGR, %/day) were calculated according to equations (1)–(3). While, fitness indexes such as condition factor (K-factor) and hepatosomatic index (HSI) were calculated according to equations (4) and (5):

$$\text{TF}_{\text{intake}} (\text{g} / \text{fish}) = [(\text{TF}_{\text{given}} - \text{TF}_{\text{uneaten}}) / \text{number of fish}] \quad \text{Eq 1}$$

$$\text{RWG} (\% / \text{g}) = [(\text{BW}_f - \text{BW}_i) / \text{BW}_i] \times 100\% \quad \text{Eq 2}$$

$$\text{SGR} (\% / \text{day}) = [(\ln \text{BW}_f - \ln \text{BW}_i) / t] \times 100\% \quad \text{Eq 3}$$

$$K - \text{factor} = (\text{BW}_f / \text{L}^3) \times 100\% \quad \text{Eq 4}$$

$$\text{HSI} = (\text{L} / \text{BW}_f) \times 100\% \quad \text{Eq 5}$$

Where; TF_{given} : total feed given (g), $\text{TF}_{\text{uneaten}}$: total uneaten feed (g), BW_f : final body weight (g), BW_i : initial body weight (g), t : time period, L^3 : total length (cm) and L: liver weight (g).

2.4. Respirometry assay

After 20 days of exposure, fish were subjected to respirometry assay. Respirometry assay was performed in four separate series of following treatments such as $\text{N}_{28^\circ\text{C}}$, $\text{L}_{28^\circ\text{C}}$, $\text{N}_{32^\circ\text{C}}$ and $\text{L}_{32^\circ\text{C}}$. Ten fish were introduced into 3.8L respirometry chamber individually for 2 h prior to measurement with continuous aeration and new oxygenated water supply. The respirometry chambers were made by a clear acrylic cylinder at a diameter of 15 cm. At measurement, aeration and water flow were discontinued, initial dissolved oxygen reading ($\text{O}_2 \text{ i}$) and initial water samples of 3 ml were taken before the chamber was sealed. Measurements were performed for 2 h and final dissolved oxygen reading ($\text{O}_2 \text{ f}$) for metabolic oxygen intake (MO_2) and 3 ml final water samples were taken for total ammonia excretion (T_{amm}) [87]. During 2 h measurement, randomly individuals were selected to count ventilation frequency at 60 min. The water sample for total ammonia excretion (T_{amm}) was kept at -80 °C and being analyzed within 24 h.

2.5. Sampling procedure

After completion of the respirometry assay, fish was anaesthetised individually with neutralised clove oil at 0.5 ml/L until fish lost their equilibrium. Fish was blotted dry followed by biometric measurement, blood was withdrawn within 1 min at the caudal peduncle using a heparinised syringe and then immediately centrifuged for 1 min at 13200 rpm at 4 °C [88]. The aliquots of plasma were immediately transferred into a new sample tube, kept under –80 °C till further analysis. After blood withdrawal, the pithing method was performed to break the spine. Muscle and liver tissues were collected on ice chilled dissection plate [89,90]. The collected samples were then labelled and kept under –80 °C for biochemistry analysis.

2.6. Metabolic oxygen intake (MO_2), total ammonia (T_{amm}) excretion and ammonia quotient (AQ)

MO_2 were calculated according to equation (6) [89,90,91] while T_{amm} excretion in the water samples was determined spectrophotometrically followed the phenol-nitroprusside method [92].

Ammonia quotient (AQ) was calculated according to equation (8) to determine the maximum protein contribution during aerobic metabolism:

$$MO_2 = (\Delta O_{2I} - \Delta O_{2F}) \times V \times 1000 \times (1 / O_{2MW}) \times (1 / BW) \times T \quad \text{Eq 6}$$

Where, O_{2I} is initial oxygen concentration (mg/L); O_{2F} is final oxygen concentration (mg/L), V is total water volume in respirometer; O_{2MW} is molecular weight of oxygen (32); BW is body weight (g) and T is time by hour (h) and unit was expressed as $\mu\text{mol/g/h}$.

$$T_{amm\text{excretion}} = (\Delta NH_{4F}^+ - \Delta NH_{4I}^+) \times V (1 / NH_{4MW}^+) \times (1 / BW) \times (1 / T) \quad \text{Eq 7}$$

Where, NH_{4I}^+ is initial ammonia concentration (mg/L); NH_{4F}^+ is final ammonia concentration (mg/L); V is total water volume in respirometer; NH_{4MW}^+ is molecular weight of ammonia (17); BW is body weight (g); T is time by hour (h) and unit was expressed as $\mu\text{mol/g/h}$.

$$AQ = T_{amm} / MO_2 \quad \text{Eq 8}$$

Where, T_{amm} is metabolic ammonia excretion; MO_2 is metabolic oxygen intake.

2.7. Plasma osmolality and electrolytes

Plasma osmolality was measured within 24 h after sample collected by using osmometer (Osmometer Advanced instrument Inc, Model 3320) with unit was expressed as mOsm/kg. While plasma electrolytes of cation (Na^+ and K^+) and anion (Cl^-) were measured using Ion Chromatography Analyzer (Metrohm 81 Compact IC Plus, Model 883) with unit were expressed as mmol/L.

2.8. Total protein, glycogen and lipid

Tissue liver and muscle were homogenized using handheld homogenizer (Kinematica Polytron™ PT1200E, Switzerland) in ice-chilled condition bolder plate before proceeding with the total protein, glycogen, and lipid analysis. For the total protein, Bradford method was performed using bovine serum albumin (BSA) as standard references [93]. While glycogen was determined by using Anthron method with glycogen as standard references [94] and lipid was extract using methanol-chloroform with tripalmitin as standard references [95]. Measurement was performed by using 96 wells microplate reader (Multiscan™ FC, Thermo scientific, US).

2.9. Statistical analysis

In order to quantify the results obtained, normality and equality of variance of sample for TF_{intake} , RWG, SGR, K-factor, MO_2 , T_{amm} , osmolality, plasma electrolyte, muscle and liver protein, glycogen and lipid were checked using Shapiro-Wilk test prior to analysis. As Shapiro-Wilk test showed homogeneity of variance ($P > 0.05$), significant differences among experimental conditions ($N_{28^\circ C}$, $N_{32^\circ C}$, $L_{28^\circ C}$ and $L_{32^\circ C}$) were performed by One-way Analysis of Variance (ANOVA) at 95 % confident limit ($P < 0.05$), then followed by multi-comparison Tukey post-hoc test. The interaction effects between temperature and pH measurement variables were analyzed using Multivariate analysis of variance (MANOVA). All statistical analyses were performed using SPSS Statistics 21.0. All data were expressed as mean \pm standard error (SE).

3. Results

3.1. Feeding, growth performances and fitness index

Hoven's carp feeding intake was higher under neutral pH than low pH condition. The highest total feed intake was recorded at high temperature ($N_{32^\circ C}$) followed by $N_{28^\circ C}$ ($P < 0.05$; Table 1). Whereas, Hoven's carp exposed under low pH ($L_{28^\circ C}$ and $L_{32^\circ C}$) showed significant reduction in the total feed intake by 63.30 % and 30.60 % compared to Hoven's carp exposed under neutral pH ($P < 0.05$;

Table 1). However, there is a double increment of feeding intake of Hoven's carp under low pH at high temperature compared to low pH at normal temperature.

Hoven's carp exposed to L_{28°C} had a significantly lowest value of relative weight gain (RWG) compared to other groups ($P < 0.05$; Table 1). The highest RWG was recorded under N_{28°C}. However, there was no significant difference in the RWG of Hoven's carp under N_{28°C} compared to high temperature groups (N_{32°C} and L_{32°C}) ($P > 0.05$; Table 1). Likewise, a trend was noted for SGR with the lowest value noticed under L_{28°C} ($P < 0.05$; Table 1) and the highest SGR was observed under N_{28°C}, followed by N_{32°C} and L_{32°C} ($P > 0.05$, Table 1).

The highest K-factor for Hoven's carp was noted under N_{28°C} and the lowest K-factor was noticed in the Hoven's carp exposed to L_{32°C}, respectively ($P < 0.05$; Table 1). Overall, low K-factor was noticed at high temperature groups (N_{32°C} and L_{32°C}). For the hepatosomatic index (HSI), no significant differences were noticed for all groups ($P > 0.05$; Table 1). Nevertheless, numerically, higher HSI value was observed in Hoven's carp exposed under neutral pH with N_{32°C}, followed by N_{28°C}. HSI for Hoven's carp exposed under low pH (L_{28°C} and L_{32°C}) were reduced by 12.83 and 7.14 %. ($P > 0.05$; Table 1). For survival rate, there was no mortality recorded during the experimental period (Table 1). Overall, significant interaction effects between temperature and pH on RWG, SGR and K-factor were observed (Table 2).

3.2. Tissue energy mobilization pattern

Muscle protein was maintained relatively stable for the Hoven's carp under neutral pH (N_{28°C} and N_{32°C}) at 221.44 ± 6.02 mg/g wet tissue and 218.53 ± 7.95 mg/g wet tissue ($P > 0.05$; Fig. 1a). While for the acidic group (L_{28°C} and L_{32°C}), muscle protein for Hoven's carp was recorded at 13.56 % and 5.50 % lower with only 191.41 ± 4.76 mg/g wet tissue and 209.27 ± 4.89 mg/g wet tissue compared to the neutral pH groups (N_{28°C} and N_{32°C}) ($P < 0.05$; Fig. 1a). In contrast, liver protein was higher in Hoven's carp exposed to high temperature groups (L_{32°C} and N_{32°C}) with about 18.14 % and 9.86 % at 140.88 ± 2.51 and 131.02 ± 2.76 mg/g wet tissue compared to N_{28°C}. Nevertheless, the lowest liver protein level was noticed in Hoven's carp exposed to L_{28°C} with a reduction of 21.31 % at 93.16 ± 2.14 mg/g wet tissue compared to N_{28°C} ($P < 0.05$; Fig. 1d). Nonetheless, interactive effect between temperature and pH was significant only in liver protein ($P < 0.05$; Table 2).

Muscle glycogen level was higher in Hoven's carp exposed to high temperature (L_{32°C}) at 21.23 ± 0.10 mg/g wet tissue followed by N_{32°C} at 21.12 ± 0.16 mg/g wet tissue, L_{28°C} at 20.55 ± 0.04 and N_{28°C} at 20.72 ± 0.04 mg/g wet tissue, respectively ($P < 0.05$; Fig. 1b). While for liver glycogen, L_{32°C} and N_{32°C} have shown increased content of liver glycogen at 46.80 ± 0.28 and 46.78 ± 0.35 mg/g wet tissue, which were 4.84 and 4.87 % higher than normal temperature (N_{28°C}) ($P < 0.05$; Fig. 1e). However, both normal temperature groups (N_{28°C} and L_{28°C}) remain relatively stable at 44.62 ± 0.29 mg/g wet tissue and 44.79 ± 0.62 mg/g wet tissue ($P > 0.05$; Fig. 1e). Therefore, interactive effect between temperature and pH was not significant in both glycogen muscle and liver ($P > 0.05$; Table 2).

There were no significant differences between lipid muscle in Hoven's carp under high temperature (N_{32°C} and L_{32°C}), which were reported at 47.65 ± 0.98 and 44.70 ± 0.69 mg/g wet tissue ($P > 0.05$; Fig. 1c). On the other hand, muscle lipid of Hoven's carp exposed under normal temperature (L_{28°C} and N_{28°C}) was relatively stable at 42.25 ± 0.60 and 43.05 ± 0.96 mg/g wet tissue. Higher liver lipid content was observed under high temperature exposure (L_{32°C} and N_{32°C}) and also under low pH at normal temperature (L_{28°C}) at 184.23 ± 7.67 , 194.03 ± 3.42 , 177.07 ± 10.63 mg/g wet tissue compared to normal temperature which was recorded at 132.79 ± 4.57 mg/g wet tissue ($P < 0.05$; Fig. 1f). Interactive effect between temperature and pH was found significant for liver lipid ($P < 0.05$; Table 2).

3.3. Metabolic oxygen intake (MO₂), total ammonia excretion (T_{amm}), ventilation frequency and ammonia quotient (AQ)

Under low pH (L_{28°C}), T_{amm} level was slightly declined by 7.40 % at 0.044 ± 0.006 $\mu\text{mol/g/h}$ but increased significantly to 34.01 % under N_{32°C} at 0.064 ± 0.004 $\mu\text{mol/g/h}$. The increase of T_{amm} was more profound under L_{32°C} to about 96.33 % at 0.094 ± 0.006 $\mu\text{mol/g/h}$ when compared with N_{28°C} at only 0.048 ± 0.003 $\mu\text{mol/g/h}$ ($P < 0.05$; Fig. 2c). A similar trend was noticed for the MO₂ (Fig. 2a), where the values maintained at 5.58 ± 0.37 $\mu\text{mol/g/h}$ and 4.96 ± 0.24 $\mu\text{mol/g/h}$ when Hoven's carp was exposed to normal temperature either neutral pH (N_{28°C}) or low pH (L_{28°C}), respectively. The MO₂ was found to increase by 18.52 % at 6.61 ± 0.36 $\mu\text{mol/g/h}$ under N_{28°C}. The highest uptake of MO₂ was recorded under L_{32°C} at 9.67 ± 0.43 $\mu\text{mol/g/h}$ ($P < 0.05$; Fig. 2a). For ammonia quotient (AQ), no significant effect was noticed but overall, Hoven's carp that were exposed to high temperature exhibited

Table 1

Feeding, growth performance and fitness indexes of Hoven's carp exposed to interactive temperature and water pH for 20 days.

Parameters	L _{28°C}	L _{32°C}	N _{28°C}	N _{32°C}
TF _{intake} , g/fish	6.14 ± 0.31^a	11.61 ± 0.50^b	16.73 ± 0.66^c	18.81 ± 0.47^c
RWG, %/g	26.18 ± 2.99^a	65.92 ± 1.72^b	74.82 ± 4.08^b	66.03 ± 2.49^b
SGR, %/day	1.15 ± 0.12^a	2.66 ± 0.05^b	2.93 ± 0.13^b	2.67 ± 0.08^b
K-factor	1.34 ± 0.05^a	1.16 ± 0.04^b	1.90 ± 0.05^c	1.27 ± 0.04^{ab}
HSI	1.30 ± 0.13	1.39 ± 0.05	1.49 ± 0.09	1.66 ± 0.05
Survival rate, %	100	100	100	100

* Lower case letter denotes significant differences within all exposure groups. Values are means \pm S.E.

Table 2
Significant level of the impact of interactive temperature and water pH for 20 days in Hoven's carp.

Parameters	Temperature		pH		Temperature × pH	
	F value	P value	F value	P value	F value	P value
TF _{intake} , g/fish	15.649	0.000	86.884	0.000	3.155	0.080
RWG, %/g	25.747	0.000	63.897	0.000	63.278	0.000
SGR, %/day	32.464	0.000	69.138	0.000	68.877	0.000
K-factor	90.453	0.000	59.627	0.000	27.637	0.000
HSI	1.792	0.190	6.078	0.019	0.194	0.662
T _{amm} , μmol/g/h	46.053	0.000	7.323	0.010	11.798	0.002
MO ₂ , μmol/g/h	92.048	0.000	26.158	0.000	30.195	0.000
Ventilation freq, no/min	5.297	0.027	31.906	0.000	15.420	0.000
AQ	2.479	0.124	0.083	0.775	0.005	0.946
Muscle protein, mg/g wet tissue	1.493	0.230	10.331	0.003	2.885	0.098
Muscle glycogen, mg/g wet tissue	14.242	0.001	3.621	0.070	0.002	0.963
Muscle lipid, mg/g wet tissue	18.736	0.000	5.339	0.027	1.748	0.195
Liver protein, mg/g wet tissue	84.528	0.000	6.294	0.020	30.890	0.000
Liver glycogen, mg/g wet tissue	19.799	0.000	0.037	0.850	0.025	0.876
Liver lipid, mg/g wet tissue	23.023	0.000	5.850	0.023	14.393	0.001
Osmolality, mOsm/kg	10.249	0.003	0.768	0.387	3.700	0.063
Na ⁺ , mmol/l	0.385	0.540	1.625	0.212	2.541	0.121
Cl ⁻ , mmol/l	0.583	0.453	1.634	0.213	2.621	0.119
K ⁺ , mmol/l	2.602	0.126	1.182	0.293	0.398	0.537

higher AQ compared to normal temperature ($P > 0.05$; Fig. 2d).

Hoven's carp exposed to N_{32°C} remained with the highest ventilation rate at 30.92% compared to N_{28°C}. For low pH group exposed to high temperature, the ventilation rate was lower compared to N_{32°C}. However, both acid groups (L_{28°C} and L_{32°C}) had no significant differences when compared to the control (N_{28°C}, $P < 0.05$; Fig. 2b). For ammonia quotient (AQ), no significant value was observed, but overall, Hoven's carp that was exposed to high temperature shown higher AQ compared to normal temperature ($P > 0.05$; Fig. 2d). Thereby, significant interactive effects between temperature and pH were observed on T_{amm}, MO₂ and ventilation frequency ($P < 0.05$; Table 2) except for AQ.

3.4. Plasma electrolytes

The highest osmolality was observed in the high temperature group under low pH (L_{32°C}) at 253.9 ± 5.82 mOsm/kg, followed by neutral pH (N_{32°C}) at 233.50 ± 4.79 mOsm/kg. There were no significant differences between normal temperature groups for both neutral pH and low pH, with osmolality recorded at 224.19 ± 10.41 and 216.56 ± 8.24 mOsm/kg, respectively (Fig. 3a). Nevertheless, for plasma electrolytes, the Na⁺, Cl⁻ and K⁺ were not significantly different among all groups ($P > 0.05$) with Na⁺ maintained at a range of 91–103 mmol/l ($P > 0.05$; Fig. 3b), Cl⁻ was between 80 and 91 mmol/l ($P > 0.05$; Fig. 3c) and K⁺ was within 14–17 mmol/l ($P > 0.05$; Fig. 3d). The interactive effect between temperature and pH was not significant for all plasma electrolytes ($P > 0.05$; Table 2).

4. Discussion

4.1. Growth and energy mobilization pattern

Hoven's carp exposed to low pH (L_{28°C}) experienced feeding suppression that led to poor in growth performances. Loss of appetite was a sign of stress in fish and consequently leading to a decrease in energy available for growth [96,97]. The poor growth performances of Hoven's carp exposed to L_{28°C} was also because of protein mobilization from both of liver and muscle tissues for metabolism purposes instead of growth. As previously reported, growth depletion occurred due to low pH stress was in associated with extra energy expenses needed to maintain basal metabolism needs as a cost of living rather than for growth [91]. Low pH resulted in poor growth performance had also been recorded in other freshwater species, including Mosambique tilapia (*Tilapia mossambica*) [98], Atlantic salmon (*Salmo salar*) [54], Nile tilapia (*Oreochromis niloticus*) [99], silver catfish (*Rhamdia quelen*) [100,101], common carp (*Cyprinus carpio*) [102] and spangled perch (*Leiopotherapon unicolor*) [103].

Whereas, Hoven's carp exposed to high temperature (N_{32°C} and L_{32°C}) shown an increase in feeding intake to gain maximize nutrients intake from dietary consumption in order to support high metabolic cost demand for living. At the same time, part of the energy obtained from dietary was deposited in liver such as protein, glycogen and lipid (Fig. 1d, e and f). The ability of energetic substrates such as protein, lipid and glycogen to be mobilized and fuel metabolic needs differs among species and influenced by environmental conditions, which affect the availability and quality of ingested food as well as energetic reserves in the body to maintain body functionality [104,105]. With that, this study revealed that liver lipid was the predominant energy utilized by Hoven's carp in normal condition (N_{28°C}). As an omnivorous fish, Hoven's carp has the advantage to mobilize energy other than protein, where they have a low dietary protein requirement compared to carnivorous species that required higher protein [68,106,107]. The

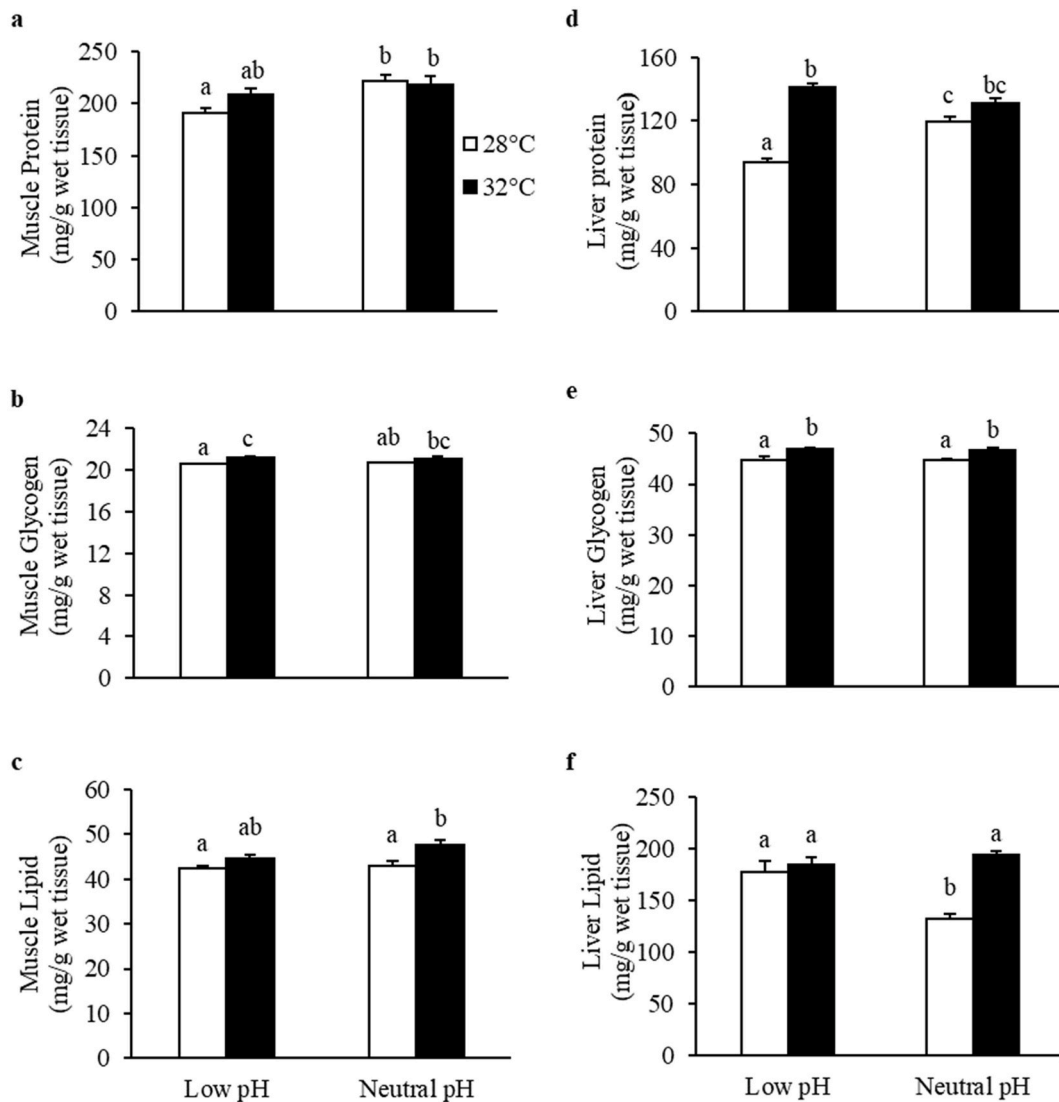


Fig. 1. Energy mobilization (mg/g wet tissue) of (a) protein, (b) glycogen, (c) lipid content in muscle (d) protein, (e) glycogen and (f) lipid content in liver of Hoven's carp exposed to interactive temperature and water pH for 20 days. Lower case letter denotes significant differences within all exposure groups. Values are means \pm S.E.

utilization of non-protein sources from lipid is beneficial for protein sparing, as lipid would provide a significant portion of the energy. At the same time, it allows the dietary protein to be used for protein synthesis and somatic growth [108,109] and probably less nitrogenous waste is being produced [110]. Several fish species such as bagrid catfish (*Pseudobagrus fulvidraco*) [111], tilapia (GIFT) (*Oreochromis niloticus*) [112] and hybrid Tambatinga ($\text{♀Colossoma macropomum} \times \text{♂Piaractus brachypomus}$) [113] have been reported to use lipid as the main energy source for basal metabolism and reserved protein for growth. However, when exposed to high temperature and low pH, Hoven's carp starts to spare liver lipid at approximately 33–46 %, which was believed as a strategy to cope with unfavorable conditions for long-term energy mobilization purpose [114]. Where, lipid is known to contain twice the energy of carbohydrates and protein for metabolic needs [115]. The mobilization of lipid under unfavorable condition was reported to be related to temperature, food availability or strenuous activity [116].

In parallel with feeding suppression, Hoven's carp mobilized liver and muscle protein when exposed to low pH ($L_{28^{\circ}\text{C}}$ and $L_{32^{\circ}\text{C}}$) prioritizing the basal metabolism needs rather than for growth. Under insufficient dietary intake, protein was accessed from reserve source to support body needs for maintenance needs in Hoven's carp. Protein intake metabolism is important by breaking the protein into amino acids carried by blood and circulated throughout the body to build protein blocks, enzymes and hormones production [117], while excess protein was catabolized in liver for energy production or converted into glucose, ketone or lipid [118]. Numerous studies have highlighted that protein mobilization occurred when glycogen and lipid reserves reached a crucial threshold [119,120,121,122,123]. However, the degradation of protein reserves was necessary to mitigate stress as muscle proteins are being mobilized as

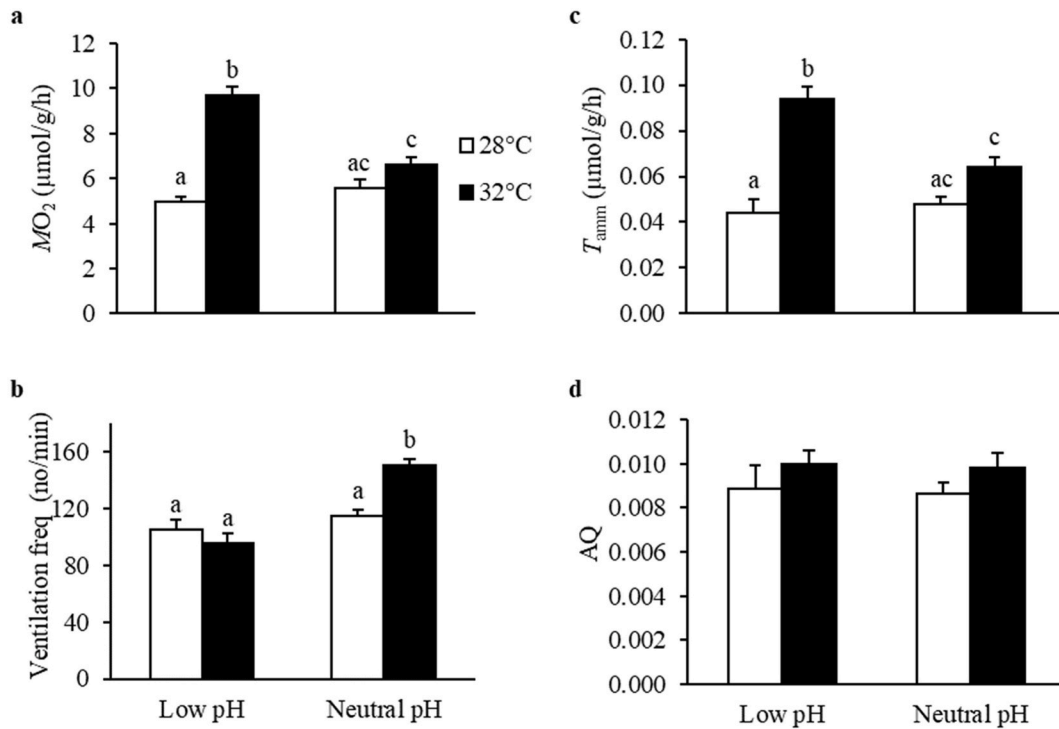


Fig. 2. (a) Total ammonia (T_{amm}) excretion, (b) Ventilation frequency (c), metabolic oxygen intake (MO_2) and (d) ammonia quotient (AQ) of Hoven's carp exposed to interactive temperature and water pH for 20 days. Lower case letter denotes significant differences within all exposure groups. Values are means \pm S.E.

a gluconeogenic substrate to increase proteolytic activity and provide sufficient amino acids for metabolic needs [124,125,126].

Differently, when exposed to higher temperature, Hoven's carp seems to restore glycogen via breakdown from protein reserves or dietary protein intake. The restoration of glycogen was essential to ensure Hoven's carp able to access readily energy when stress by converting glycogen to glucose through glycogenolysis from protein or lipid to satisfy energy demand [121,125]. While, the excess glucose from ingested carbohydrates was also deposited in the liver as glycogen through glycogenesis or converted to lipid through lipogenesis [127].

4.2. Osmorespiration strategy

Hoven's carp in the control group were able to maintain the MO_2 and T_{amm} excretion when exposed to low pH at normal temperature (L_{28}). Maintaining a stable osmorespiration under L_{28} was a strategy of energy conservation due to low feed intake and endogenous ammonia accumulation. Small amount of ammonia might be derived from catabolism of reserved protein for energy production but was able to be excreted. This explained the energetic trade-off for growth by reallocating the energy expenditure for basal metabolic at low metabolism. Wood [128] mentioned that the alteration of T_{amm} excretion was associated with protein catabolism in corresponding with the dietary protein levels. Our results have shown that Hoven's carp exposed to a combination of low pH and high temperature (L_{32}) had the highest MO_2 and T_{amm} excretion rates which increased to 2-folds higher than normal temperature (L_{28} and N_{28}) (Fig. 2a and c). The increased MO_2 with reduced ventilation under low pH-high temperature (L_{32}) group indicated that Hoven's carp intensified the oxygen intake in order to support all biological processes that included specific dynamic action, digestion, ingestion with high feed intake volume and basal metabolic needs. But at the same time, Hoven's carp reduced its ventilation frequency to minimize the contact of gills with low pH water that may disturb osmorespiration. Thereby, Hoven's carp remodeled their gills phenotypes to enhance osmorespiration performance by increasing oxygen uptake per volume of respiration [129]. This was proven recently by Mohamad et al. [62] that under low pH (pH 5), Hoven's carp had a minimal effect on respiration and excretion to cope with the osmorespiration requirement via gill filament microstructural modification. Another possibility would be enhanced blood-oxygen carrying capacity by increased water volume into gills buccal cavity per respiration volume by changing the ventilation strategy into high amplitude-low frequency under resting condition. However, the haematology analysis was not carried out in this study. This speculation contradicts the previous reports which stated that freshwater fish exposed to low pH caused haemoglobin dysfunctions [130,131] that limits blood-oxygen carrying capacity and reduced oxygen intake performance [132]. However, there are findings showed a notable increase of MO_2 under low pH environment on other species as well such as bathypelagic mysid *Gnathopausia ingens* [133], common carp (*Cyprinus carpio*) [134], slangled perch (*Leiopotherapon unicolor*) [135] and sea urchin (*Heliocidaris erythrogramma*) [136]. Therefore, we suggested that the ability to modify the osmorespiration pattern under low pH was

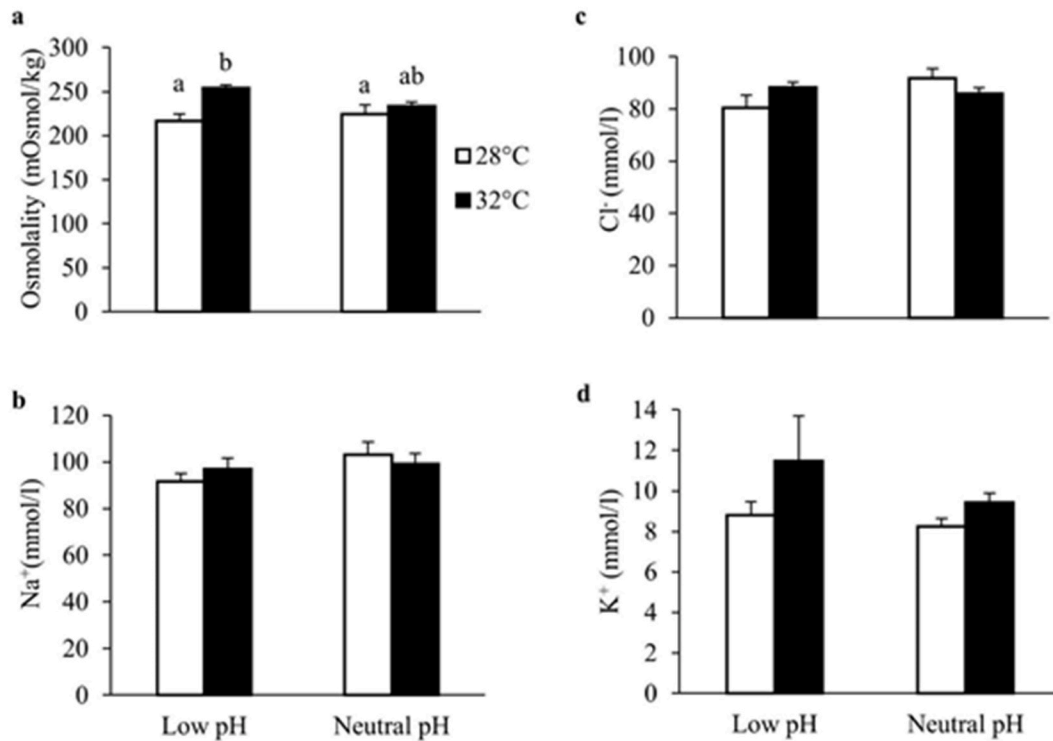


Fig. 3. (a) Osmolality (mOsm/kg) and electrolytes (mmol/l), (b) sodium, (c) chloride and (d) potassium in plasma of Hoven's carp exposed to interactive temperature and water pH for 20 days. Lower case letter denotes significant differences within all exposure groups. Values are means \pm S.E.

species-dependent.

In addition, high osmoregulation cost was in association with an increase in feed intake to support high metabolic needs (Table 1). This was proven with the high oxygen intake that was necessary to facilitate aerobic metabolism to breakdown the macronutrients intake into smaller molecules of amino acid, lipid and glucose as accessible energy sources (ATP). As consequences, breakdown of protein also generated nitrogen waste with ammonia as a by-product to be excreted (Fig. 2c). Our result was in agreement with previous reports noted that higher ammonia excretion was correlated with the digestion and breakdown of protein intake [137,138], which was found in rainbow trout (*Oncorhynchus mykiss*) [139,140], common carp (*C. carpio*) [141,89], marble goby (*Oxyeleotris marmorata*) [82] and hybrid grouper [91]. Ammonia is known as toxic to fishes, therefore the ammonia needs to be excreted to prevent ammonia intoxication [142]. Under low pH condition, ammonia excretion across the branchial epithelium via passive NH₃ diffusion and coupled with active transport through exchangers and transporters such as Rhcg, V-type H⁺-ATPase, Na⁺/H⁺ exchanger NHE-2 and/or NHE-3, Na⁺ channel to facilitate the excretion process [143,144,145,146]. Whereby, low pH condition is known to favor NH₃ diffusion across gills epithelium and facilitate rapidly trapped with H⁺ to form NH₄⁺ in acidic water [147,148]. For example, zebrafish (*Danio rerio*) [149] and common carp (*C. carpio*) [57] showed an activation of NHE in response to increased ammonia excretion under low pH environment. Nevertheless, the ammonia excretion rate measured in this study was total ammonia (NH₃ + NH₄⁺), hence the proportional ratio between the interchangeable NH₃ and NH₄⁺ was uncertain since plasma pH was not measured in this study.

On the other hand, we also noticed that Hoven's carp exposed to N_{32°C} had a higher ventilation frequency. Hyperventilation under N_{32°C} was because of low oxygen solubility and availability [150], thereby Hoven's carp increased ventilation rate to enhance oxygen intake by increasing water flow to the gills [151] per breath volume [129] with gill remodeling [62,152]. Nevertheless, the decreased ventilation frequency was observed under low pH conditions (L_{28°C} and L_{32°C}), which likely owes to the fish's response to reduce the cost of osmoregulatory by limiting the contact of the gill surface with acidic water. Low pH altered gill phenotypic with lesion and thickness secondary lamellae of the gill of Hoven's carp was noticed previously [62], but still able to osmoregulate for basal metabolic needs. Low pH not only reduce gill ventilation has been seen in Amazonia fish (*Serrasalmus eigenmanni*) but also to reduce effluxes of Na⁺ and Cl⁻ under hypoxia [153]. As we know, acidic water alters ionoregulatory by inhibiting Na⁺ and Cl⁻ uptake and stimulates passive diffusive effluxes of these ions as reported in common carp (*C. carpio*) [56], zebrafish (*D. rerio*) [154], angelfish (*Pterophyllum scalare*) [63]. However, our findings also revealed that Hoven's carp was able to maintain a stable plasma Na⁺, Cl⁻ and K⁺ in all groups, this indicated that feed intake has sufficiently restored the plasma electrolytes with stable plasma osmolarity level. Maintaining a stable plasma osmolarity was important to facilitate metabolites mobilization to support basal metabolic needs. Compensation of electrolytes loss through feeding until satisfaction has been demonstrated in rainbow trout (*Oncorhynchus mykiss*) [155,156] and common carp (*C. carpio*) [88] as well as dogfish (*Squalus acanthias*) [90].

5. Conclusion

Low pH suppressed growth performance and altered protein mobilization to support basal metabolism. When exposed to high temperature, Hoven's carp increased feed intake to secure sufficient energy intake for metabolism, energy reserve and growth purposes. As a conclusion remark, Hoven's carp remodeled their energy mobilization pattern and osmorepiration scope to cope with the changing environment for growth and survival.

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Additional information

No additional information is available for this paper.

Ethical statement

Experimental procedures, animal handling and dissection method used in this experiment followed the Animal Ethic Guidelines approved by the Committees of Ethic Animal Care, Universiti Malaysia Terengganu (UMT/JKEPHT/2019/41).

Data availability statement

The data that support the findings of this study are available on request from the corresponding author.

CRedit authorship contribution statement

Sharifah Rahmah: Writing – review & editing, Validation, Supervision, Formal analysis, Conceptualization. **Mohamad Jalilah:** Project administration, Formal analysis. **Mazlan Abd Ghaffar:** Writing – review & editing, Funding acquisition. **Leong-Seng Lim:** Writing – review & editing, Software. **Li Qun Liang:** Writing – review & editing, Investigation, Conceptualization. **Hon Jung Liew:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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