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High environmental temperature and low pH stress alter the gill phenotypic plasticity of Hoven's carp *Leptobarbus hoevenii*

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ABSTRACT

Climate warming and low environmental pH are known to negatively impact aquatic organisms from cellular to organismal and population levels. For ammonotelic freshwater species, any fluctuation in abiotic factors will cause disturbance to the fish, specifically at the gills which act as a multifunctional organ to support all biological processes. Therefore, this study was designed to investigate the effect of temperature (28 versus 32°C) and pH (7.0 versus 5.0) stress on the gill plasticity of Hoven's carp after 20 days of exposure. Results demonstrated that high temperature and low pH caused severe changes on the primary and secondary lamellae as well as the cells within lamellae. An increasing trend of the proportion available for gas exchange (PAGE) was noticed at high temperature in both pH exposures, which resulted from a reduction of the primary lamellae width with elongated and thinner secondary lamellae. Gill damage was severe at high temperature with aneurysm, oedema, hypertrophy, curling of secondary lamellae and epithelial lifting. Under acidic pH at ambient water temperature Hoven's carp showed aneurysm, oedema, hyperplasia and lamellae fusion. We can conclude that even though Hoven's carp showed some gill remodeling to adjust to the changing environment, tissue damage was still substantial after 20 days of exposure.

Keywords: Acidification, aquaculture, branchial, climate change, freshwater fish, histopathology, osmoregulation, remodeling

1. INTRODUCTION

Rapid modernization leads to the increase of atmospheric CO_2 that contributes to worldwide rising temperatures and ocean acidification (Shi, 2003; Cherubini et al., 2011; Martínez-Zarzoso and Maruotti, 2011; Bony et al., 2013; Miller et al., 2017; Manabe, 2019). Climate warming poses a major threat to all life forms. This is especially true for aquatic organisms as temperature does not only affect the chemical equilibrium of the natural ecosystem, but also influences the physiological and biochemical processes within aquatic organisms (Somero, 1997, 2004; Pörtner et al., 2006). For example, species living in the tropical regions are accustomed to a narrow thermal niche, thereby limiting their thermal acclimation capacity. Thus, tropical animal species are more vulnerable towards temperature fluctuations in contrast to temperate species that are well adapted to annual temperature changes following seasonal cycles (Janzen, 1967; Vinagre et al., 2015; 2016). Being unable to acclimatize to the changing temperature could pose serious issues to an organism as an optimum temperature is crucial to maintain basal metabolic needs and homeostasis (Evans et al., 2005).

Acidification of freshwater occurs either naturally by organic acid originating from decomposed matter (Holland et al., 2012; 2014) or through continuous anthropogenic gaseous emission of e.g. sulfur dioxide, nitrogen oxides, and ammonia (Driscoll et al., 2003; Duan et al., 2016; Jia and Gao, 2017). In addition, other contributors of acidification include aquaculture (Biermann et al., 2019) and agriculture activities (Huang et al., 2017), and municipal and industrialization effluent discharge (Naigaga et al., 2011; Nyuyen et al., 2019). All these processes impose detrimental effects on species diversity and ecosystem health (Mills et al., 2000; Petrin et al., 2008, Jellyman and Harding, 2014). Additionally, acidification accelerates leaching and increases the mobility of metals (e.g. Al, Pb, Cu, Zn, Fe) (Campbell and Stokes, 1985; Greig et al.,

2010; Hogsden and Harding, 2012). As the acid deposition may exceed the aquatic environment's natural acid buffering ability, the acid-base chemistry will be altered and the elevated H^+ deposition will result in low pH (Norton and Veselý, 2003) which will affect aquatic organisms.

In general, most fish's gills consist of four pairs of gill arches with on each gill arch two rows of filaments or "primary lamellae". These primary lamellae are equipped with numerous secondary lamellae rich in blood capillaries (Hurges and Morgan, 1973; Evans et al., 2005). They are considered as the largest organ that interacts directly with external environment with a high surface area about 0.1–0.4 m^2/kg body weight. Fish gills are known for their multiple functions, serving as primary site for respiration (Koppang et al., 2015), ionic and acid-base regulation (Evans, 2010), osmoregulation (Rombough, 2007), nitrogenous waste excretion (Ip and Chew, 2010). Gill functioning is under hormonal regulation (Evans et al., 2005), e.g. it is well documented that the adenohypophysial peptide and prolactin play a major role in osmoregulation in freshwater gills (Manzon 2002). The gill epithelium is covered by different types of undifferentiated and differentiated epithelial cells, including pavement cells (PVs) and chloride cells (CCs), also referred to as mitochondria rich cells (MRCs) or ionocytes (Dymowska et al., 2012; Marshall et al., 2017). In addition, mucous cells or goblet cells and stem cells that are essential in the regulation of physiological-biochemical activities are also found in gill epithelia (Laurent and Dunel, 1980; Dymowska et al., 2012; Koppang et al., 2015).

The commonly found cell types in gills include pavement cells, ionocytes, mucous cells and stem cells. Squamous and cuboidal pavement cells cover more than 90% of the epithelium. They generally have large and polygonal apical surface with microridges (Wilson and Laurent, 2002). Squamous pavement cells have a central small spherical nucleus, while cuboidal pavement cells have a cube-shape and a large central spherical nucleus (Wilson and Laurent, 2002).

Pavement cells act as a protective layer for innermost differentiated cells (Laurent and Dunel, 1980) and are involved in gas exchange and ion regulation (Laurent et al., 1994, Perry, 1997). Primarily found on gill filaments, the mitochondrial-rich ionocytes are known to maintain ion regulation and acid-base homeostasis (Dymowska et al., 2012). The mucous cells or goblet cells are responsible for mucus secretion that protects gill cells (Wilson and Laurent, 2002). Stem cells are classified into growth and homeostatic stem cells corresponding to their function for growth and homeostasis maintenance (Stolper et al., 2019).

Under climate warming and acidification scenarios, fish gills are the first impacted sites, as the gills are the first organ in direct contact with the surrounding water (Koppang et al, 2015). Previous studies have shown that when fish such as common carp (*Cyprinus carpio*) and rohu (*Labeo rohita*) were exposed to warm temperatures, their gill structures showed significant modification such as curling, lamellar fusion, swelling of the epithelial cells, hyperplasia, swollen bases and clubbing of secondary lamellae (Ahmad et al., 2011; Dash et al., 2011). On the other hand, acidification is known to stimulate mucus production and mucocyte proliferation with the presence of necrosis, lamellar fusion, hyperplasia, hypertrophy and enlargement of chloride cell on the gills (Reid, 1995). These alterations impair ammonia excretion (Mohammadi et al., 2019), respiration (Chevalier et al., 1985; Daye and Garside, 1976; Mueller et al., 1991), osmoregulation and acid-base regulation (Leino and McCormik, 1984; Peuranen et al., 1993; Hirata et al., 2003, Furukawa et al., 2011), and are potentially lethal in fish with low coping mechanism. Nevertheless, some species can survive at pH levels below 5.0 such as black bullheads (*Ictalurus melas*), yellow perch (*Perca flavescens*) (Rahel and Magnuson, 1983), streaked prochilod (*Prochilodus lineatus*) (Zaniboni-Filho et al., 2002), redfin bully (*Gobiomorphus huttoni*), inanga (*Galaxias maculatus*), brown trout (*Salmo trutta*), longfin eel (*Anguilla dieffenbachia*) and koaro (*Galaxias brevipinnis*)

(Jellyman and Harding, 2014). Japanese dace (*Tribolon hakonesis*) are even able to survive at pH 3.5 by positively regulating the proliferation of their chloride cells in the gills to increase ion transportation while maintaining gas exchange efficiency (Kaneko et al., 1999). Therefore, remodeling gill structures is an important strategy to cope with a changing environment in order to survive (Kaneko et al., 1999; Sollid et al, 2003; Sollid and Nilsson, 2006; Matey et al., 2011; Sinha et al, 2014).

As a valuable ornamental and food fish species with high economic values (Rohani et al., 2010, Mok et al., 2012; Xiong et al., 2015), the natural populations of Hoven's carp are already at risk due to unregulated fishing activity (Chong et al., 2010). Further deterioration in environment quality as a result of climate warming and acidification puts more stress on the already declining Hoven's carp population (Kamarudin, 2013). Still, there is a lack of information regarding gill structural modifications of Hoven's carp when coping with warming and acidifying environments. Such modifications are important to be used as early stress warning symptoms. Thus, the aim of our study was to examine the gill histopathological structural modifications of Hoven's carp exposed to several combinations of temperature (28°C vs. 32°C) and pH (pH 7 vs. pH 5) for 20 days. Our results focused on the alteration occurring in primary and secondary lamellae – the primary surfaces for gaseous exchange in fish. With the knowledge highlighted above as background, and the fact that high temperature is known to induce high metabolism, this study hypothesized that Hoven's carp gill cells would be sufficiently equipped to support osmorespiration for basal metabolism needs at high temperatures, and therefore, no gill structural changed would be observed except longer and thinner lamellae in order to increase the exchange surface and reduce the diffusion distance. However under reduced pH, we hypothesized that Hoven's carp would experience disturbances at the gill surface leading to difficulties in

osmoregulation primarily. Gill modification would be required to cope with the reduced pH, especially on primary and secondary lamellae where we expected reductions in exchange surface to reduce effects on osmoregulation. When Hoven's carp would be exposed to combined high temperature and reduced pH conditions, we hypothesized that fish would suffer more, and greater gill structural modification would be expected but it remains unclear whether this would lead to reduced or increased contact surface area with the changing external environment.

2. MATERIALS AND METHODS

2.1. Experimental animals and maintenance

A total of 40 Hoven's carp with an average body weight (BW) of 22.46 ± 0.95 g were purchased from KT Aquarium, Pet Shop, Kuala Terengganu, Malaysia. All fishes were randomly distributed equally (n = 10) into four 200 L holding glass aquarium supplied with 27-28°C dechlorinated tap water with ionic composition of 0.132 Na^+, 0.069 Cl^-, 0.037 K^+, 0.043 Ca^+ and 0.003 Mg^{2+} mmol/L in the Institute of Tropical Aquaculture and Fisheries (AKUATROP) hatchery facility, Universiti Malaysia Terengganu (UMT). Fish were fed twice a day ad libitum and allowed to acclimatize for 14 days to hatchery conditions prior experimentation with a 12h:12h (light: dark) photoperiod cycle. Each aquarium was equipped with a bio-mechanical filtration system containing wadding, bio-balls and gravel sand to ensure water conditions at optimum range. Water quality was monitored using API Freshwater Master Test Kits (MARS® Fishcare Brands, UK) with NH_3/NH^+_4 <0.25 mg/L, NO^-_2 <0.25 mg/L and NO^-_3 <20 mg/L. Dissolved oxygen was measured using a Portable Dissolved Oxygen Meter (Waterproof CyberScan DO 300, Eutech Instruments/ Oakton Instruments, USA) and maintained > 6.0 mg/L at all times. Water pH was maintained at about ±7 and monitored using an Eco pH+ meter (Trans Instruments, Singapore).

2.2. Experimental design

This experiment was planned with a 2x2 factorial design with temperature (28°C as normal temperature versus 32°C as high temperature) and water pH (pH 7 as neutral versus. pH 5 as acidic) with (A) 28°C ± pH 7 (28N; n = 10), (B) 32°C ± pH 7 (32N; n = 10), (C) 28°C ± pH 5 (28A; n = 10) and (D) 32°C ± pH 5 (32A; n = 10). Fish conditioned to 28N were referred to as control. All fish were redistributed randomly into four holding glass aquaria sized at 1.5 x 1.5 x 3.0 ft and subjected to experiment conditions for 20 days. The water temperature was chosen based on the results obtained from preliminary experiments, 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) with low water pH (5.3 ± 0.4). In order to achieve the desired water temperature and acidic water pH, temperature was gradually increased by 0.5°C daily to ± 32°C using a regulated aquarium heater (RS-978, 500W) and acidic water pH was slowly reduced at a rate about ± 0.5 unit per day to reach at about pH ± 5.0 using 1mM hydrochloric acid (HCl) controlled by a peristatic pump (SOGO, GB37-530). The pH value was monitored and recorded every 2h. Ad libitum feeding was performed twice daily at 08:30h and 17:00h with a commercial feed (TP- 1, STAR FEED) containing of 28% crude protein , 3% crude fat and 12% moisture in the diet. Weekly, 50% of water were renewed with pre-conditioned water from a stock prepared according to experimental conditions.

2.3. Sampling procedure

After 20 days of exposure, fish were anaesthetized with clove oil at 0.5 ml/l until fish lost their equilibrium and no ventilation was detected. Thereafter, fish were killed by severing the spinal

cord followed by collection of the gills (Liew et al., 2012). For gill morphometric analysis, the left side of the secondary gill was carefully procured and immediately preserved in fixative Bouins's solution at a ratio of 15:5:1 (saturated picric acid: 37% formaldehyde: glacial acetic acid) for 24h at room temperature before storing in 70% alcohol for histochemistry analysis.

2.4. Histochemistry assay

The entire second gill arch was sampled and stored in 70% alcohol individually for 24h. After 24h of fixation, gill samples were individually transferred into a labelling block before being washed and dehydrated in a series of alcohol solution (70-100%).Thereafter, the samples were cleared with xylene and infiltrated with paraffin wax for 19h using a semi-enclosed Benchtop Tissue Processor (LEICA TP 1020, USA). Samples were embedded in paraffin wax using a modular tissue embedding center consisting of a cold plate (Leica EG1150 C, USA) and heated paraffin dispensing module (Leica EG1150 H, USA). Final fixing tissue blocks were cut into 3 µm thickness with a fully automated rotary microtome (Leica RM2255, USA). The sections were stained with haematoxylin and eosin (H and E) staining solution following a standard protocol described by Hassan et al. (2013; 2015).

2.5. Gills histopathological assessment

Histopathological assessment was conducted via quantitative and semi-qualitative examination to determine the gill structural alterations on primary and secondary lamellae using a Compound Advanced Research microscope (Nikon Eclipse 80i) equipped with a Nikon Digital Sight camera (Nikon DS-Fi3). Photomicrographs from randomly selected areas of the 'mid regions' of individual fish gill filaments were taken using 10x, 20x and 60x magnification (dependent on types

of tissue examination). Basal epithelial thickness (BET), secondary lamellar length (SLL), secondary lamellar width (SLW), secondary lamellae base length (SLBL), secondary lamellar perimeter (SLP) and interlamellar distance (ID) of each filament were examined by using Imaging Image NIS-element D software (version 4.11.00 64-bit, Nikon, Japan). For BET, six measurements were measured from the basal lamina onto the middle of the cartilaginous support at both side of the primary lamellae. In addition, measurements were performed for SLL, SLW, SLP, SLBL and ID on each secondary lamellae in triplication. The proportion available for gas exchange (PAGE) were calculated as (Nero et al., 2006):

$$\text{PAGE (\%)} = \frac{\text{Mean SLL}}{\text{Mean BET} + \text{Mean SLL}} \text{ x } 100\ \%$$

Semi-qualitative examination was assessed according to Bernett et al. (1999). Ten filaments for each individual sample were selected randomly to calculate the reaction pattern (Rp) and total gill pathological index (I_{gill}). The gill pathological index (I_{gill}) was calculated as:

$$Rp = \sum_{alt} (a_{gill\ rp\ alt} \times w_{gill\ rp\ alt})$$

$$I_{gill} = \sum_{rp} \sum_{alt} (a_{gill\ rp\ alt} \times w_{gill\ rp\ alt})$$

Where, '$_{rp}$' referred as reaction pattern, '$_{alt}$' referred as alteration, 'a' referred as score value and 'w' referred as important index. Thus, the reaction patterns were categorized into three categories as Rp1 was circulatory disturbance; Rp2 was regressive change that caused cell damage and organ malfunction; and Rp3 was progressive change with increase of cells and tissue activity. '$a_{gill\ rp\ alt}$' was the score value of a reaction pattern at a specific alteration ranging from 0-6 on the degree of extent of the lesion alteration with (0) unchanged; (2) mild occurrence; (4) moderate occurrence; (6) severe occurrence. While, '$w_{gill\ rp\ alt}$' was importance factor of the reaction pattern for a specific alteration assessing how the lesion impairment might affect fish health status and categorised into

I, II and III. Where (I) referred to no alteration on functioning of the tissue which is easily reversible when the irritant exposure ends; (II) referred to moderate alteration which affects the function of the associated tissue which may or may not reversible depend on severity and degree of alteration; and (III) referred to severe alteration that causes irreparable damage and may lead to partial or total loss of organ function (Table 1).

2.6. Statistical analysis

In order to quantify the results obtained, normality and equality of variance of sample for BET, SLL, SLP, SLBL, SLW, ID and PAGE were checked using Shapiro-Wilk test prior to analysis. As Shapiro-Wilk test showed homogeneity of variance ($P>0.05$), the normality of Rp1, Rp3 and I_{gill} were checked using normal probability plots. Comparisons of groups were performed by One-way ANOVA at 95% confident limit ($P<0.05$).The significance difference among the four different factorial combinations (temperature versus pH) on BET, SLL, SLP, SLBL, SLW, ID, PAGE, Rp1, Rp3 and I_{gill} were examined by multi-comparison Turkey post-hoc test. In addition, significant different between Rp1 and Rp3 within the exposure group were examined by using unpaired student T-test at 95% confident limit ($P<0.05$). The interaction effect between temperature and pH factors on BET, SLL, SLP, SLBL, SLW, ID and PAGE were analyzed using Multivariate analysis of variance (MANOVA). All the statistical analyses were carried out using SPSS Statistics 21.0. All data were presented as mean ± standard error (SEM).

3. RESULTS

Results showed that Hoven's carp experienced gill morphology remodeling to cope with temperature and low pH challenges. Hoven's carp living under 28N exhibited normal and healthy

gill structures with cells of normal morphology arranged systematically within the lamellae (Fig. 1A). In contrast, Hoven's carp that were exposed to high temperature and/or acidic condition (32N; 28A and 32A) showed major gill structural modifications on both primary and secondary lamellae (Fig. 2, 3 and 4). The exposure of Hoven's carp to 32N and 32A significantly reduced (approximately 50%) their basal epithelial thickness (BET) compared to Hoven's carp that lived under 28N (P<0.05). Gill structure alterations were also noticed on secondary lamellae length (SLL), secondary lamellae perimeter (SLP) and secondary lamellae width (SLW) of Hoven's carp. Regardless of water pH, secondary lamellae of Hoven's carp exposed to high temperature (32N and 32A) were found to be elongated at 101.87±9.04 µm and 96.89±6.98 µm (SLL) and thinner at 4.28±0.10 µm and 5.10±0.12 µm (SLW) as compared to 28N with SLL at 74.87±2.75 µm and SLW at 6.20 ±0.14 µm, respectively. But, Hoven's carp that conditioned under 32N, have thinner secondary lamellae base length (SLBL) at 10.50 ±0.63 µm compared to fishes conditioned to 32A at 18.20 ± 1.01µm (Table 2). Thus, the proportion available for gas exchange (PAGE) became 17% and 21% higher for Hoven's carp exposed to high temperature at 32N and 32A compared to ambient temperature (28N and 28A), respectively (P<0.05; Table 1). Our results also revealed that acidity by itself induced the increase of PAGE as evident in Hoven's carp at 28A, where PAGE was 9.3% higher compared to 28N. Overall, significant interaction effects between temperature and pH effects on BET, SLBL and PAGE were observed (Table 3).

Two reaction patterns, i.e. circulatory disturbance (Rp1) and progressive changes (Rp3) were observed in all groups expect 28N (Table 4). Mild aneurysm occurred at the tip of secondary lamellae of Hoven's carp exposed to high temperature and/or acidic pH at 32N; 28A and 32A (Table 4) without any sign of rupture (Fig. 2C, 3B, 3E and 4C). Other than aneurysm, Hover's carp conditioned to high temperatures (32N and 32A) exhibited similar gill modifications such as

hypertrophy and oedema. However, the locations of oedema on the gill filaments were slightly different: at 32N it was found mainly on primary lamellae (Fig. 2D and 2E), whereas at 32A it was observed on primary and secondary lamellae (Fig. 4B). Nevertheless, the score of oedema and hypertrophy presence on Hoven's carp conditioned at 32N were higher compared to 32A (Table 4). In addition, curlings of the secondary lamellae were noticed under 32N (Fig. 2B). Meanwhile, epithelium hyperplasia was noticed in Hoven's carp exposed to 28A. This led to the fusion of several secondary lamellae due to the proliferation of epithelial cells at the base epithelium that filled the gap between interlamellar distance (Fig. 3C and 3D). Overall, Hoven's carp that were exposed to high temperature alone had a higher total gill pathological index ($P<0.05$) with Rp3 higher than Rp1 for 32N. While in acidic pH at high temperatures, total gill pathological indices were completely different with Rp1 higher than Rp3 at 32A compared to 28A with Rp1 lower than Rp3, respectively (Table 5).

4. DISCUSSION

Temperature and low pH induced significant modifications in the morphology of Hoven's carp gill filaments (Fig. 2, 3, 4 and Table 2, 3, 4). These modifications corresponded to different degrees of pathology indexes (Table 5), where hyperplasia, curling structure and fusion were pathological while other modifications such as thinner and elongated lamellae were considered as gill remodeling as a way to cope with the changing environment to ensure survival and growth. For example, it was observed that when exposed to reduced water pH at normal temperature (28A), the gill filaments of Hoven's carp showed hyperplasia at the base of the interlamellar space with epithelial cells proliferating and filling up the interlamellar space, causing fusion of the secondary lamellae. This symptom is also reported in brook trout (*Salvelinus fontinalis*) exposed to ±pH 5

*(*Muelle et al., 1991; Conklin et al, 1992). The lesion thickness caused by hyperplasia or hypertrophy negatively affects respiratory and osmoregulation routine functions due to the increased distance between the blood capillary and the lamellar surface, but positively acts as a defense mechanism against water borne harmful materials by limiting diffusion into the respiratory lamellae (Fernandes and Mazon, 2003; Camargo and Martinez., 2007; Sinha et al., 2014; Georgieva et al., 2018). However, it is believed that mild occurrence of hyperplasia and fusion had minimal effect on respiration and excretion as other areas on the secondary lamellae showed an increase of 22% and decrease of 12% in SLL and ID, respectively to cope with the osmorespiration needs (Fig. 3F and Table 2).

Also, at 32°C in both neutral and acidic pH (32N and 32A), gill remodeling led to a thinner and elongated shape of the secondary lamellae with a lessening of interlamellar distance and a reduction of basal epithelial thickness (BET) on primary lamellae. This expansion of respiratory surface area induced an increase in the PAGE, which is believed to facilitate gas exchange efficiency to provide sufficient oxygen for the increased metabolic demands at higher temperatures. However, by increasing respiratory surface area, gills will also be more exposed to unfavorable environments (Jacquin et al., 2019). Gill remodeling is necessary to strategically optimize gas exchange and at the same time maintain homeostatic balance (Liew et al., 2012). Findings in the present study – the elongation and thinning of the secondary lamellae to cope with the high oxygen demand in warming environments – are in agreement with the observations found in other cyprinid members such as crucian carp (*Carassius carassius*) and goldfish (*Carassius auratus*) (Sollid et al., 2005, Sollid et al., 2003). Throughout the study period, hypertrophy was noticed when Hoven's carp were conditioned to 32°C with the presence of oedema on primary lamellae at 32N (Fig. 2D and 2E). However, when Hoven's carp were confronted with 32A, oedema symptoms were more

perceptible and not only found on primary lamellae but also at the edge of secondary lamellae resulting in lifting of the respiratory epithelial (Fig. 4A). Such epithelium lifting is regarded as gill damage and is considered responsible for electrolyte loss (Mallat, 1985). When gill epithelial lifting occurs, this damage is difficult to reverse and cells may not be able to recover back to the normal structure (Movahedinia et al., 2012).

Increased flow rate into the blood capillaries may cause aneurysm as pillar cells are unable to support the blood flow and rupture, causing bleeding (Martinez et al., 2004). Surprisingly, aneurysm symptoms were noticed in Hoven's carp exposed to both high temperature and low pH stress (32N, 28A and 32A) with the same mild degree of lesions (Table 4). Besides aneurysm, curling of the secondary lamellae was also present when exposed to 32N, which also affects blood flow circulation (Karan et al., 1998). Aneurysm and curling of the secondary lamellae are common symptoms reported in other fish species such as snakehead (*Channa punctatus*) and iridescent shark (*Pangasianodon hypophthalmus*) exposed to high temperature and/or toxicants (Mishra and Mohanty, 2008; Das et al., 2018; Kumar et al., 2019). This strategy is believed to improve oxygen diffusion and serve as a defense mechanism to protect lamellae (Elshaer et al., 2013).

Under neutral pH condition, the surface area of ionocytes is known to change based on acid-base equivalents (H^+ and HCO_3^-) in order to maintain neutral blood pH (Perry, 1997). Previous studies reported proliferation of ionocytes in correlation with low water pH and in response to the increase in $NaCl^-$ uptake (Leino et al., 1987; Leino and McCormick, 1984; Karlsson et al.1986). Cell proliferation, like any lesion, may impede the diffusion of respiratory gases as the thickness of the epithelium increases. Generally, low pH inhibits active Na^+ uptake and reduces the gradient pressure to drive passive Na^+ influx, as observed in various fish species including in rainbow trout (*Oncorhynchus mykiss*) (McDonald et al., 1983), common carp (*Cyprinus carpio*)

(Mathan et al., 2010), angelfish (*Pterophyllum scalare*) and discus (*Symphysodon discus*) (Duarte et al., 2013). However, the effect of low pH on Na^+ uptake could be species dependent as some species such as the zebra fish (*Danio rerio*) (Kumai et al., 2011) and cardinal tetras (*Paracheirodon axelrodi*) (Gonzalez and Wilson, 2001) showed contradicting results. Goldfish exposed to high temperature were found to have a low surface area and number of ionocytes to limit Cl^- loss (Mitrovic and Perry, 2009). Nevertheless, iono-osmoregulatory disturbance caused by sensitivity to low pH (McDonald et al., 1991; Gonzalez and Wilson, 2007; Perry et al., 1992; Yanagitsuru et al., 2019) and high temperature (Houston et al., 1968; Gale et al., 2011) may differ according to species and natural habitat.

Based on the total pathological index, Hoven's carp showed higher sensitivity towards high temperature than towards pH with an index of 18.00±0.60 under neutral pH (32N) followed by high temperature under acidic pH (32A) at 12.00±0.78 and lastly ambient temperature at acidic pH (28A) at 9.75±0.25 (Table 5). However, at similar high temperatures (32 °C), the reaction patterns were different when pH was reduced, where Hoven's carp exposed to 32A suffered from circulation disturbance, while at 32N, increased cell and tissue activity to facilitate iono-osmoregulation was noticed. Modifications of gill filament structure such as BET, SLL, SLP, SLBL, SLW and ID indicate a physiological adjustment strategy to facilitate an osmorespiratory compromise. Based on interactive analysis it was shown that BET, SLBL and PAGE were highly affected by the combined stress of temperature and low pH exposure (Table 3). As we known, both BET and SLBL functions include metabolic waste exchange, uptake of essential ions and oxygen transport. This was paralleled with the finding that PAGE was also significantly affected by the interactive impact of temperature and reduced pH. Nevertheless, this did not lead to lethal

effect to Hoven's carp, as no mortality was noticed throughout the exposure period. Therefore, this indicated that Hoven's carp was able to cope with the exposure conditions within this time frame.

It is important to note that the current study focused only on the general filament morphology, and the characteristics of epithelial cells (e.g.; ionocytes, mucus cell, pavement cell) should also be investigated in future studies to describe the functional and mechanistical modifications of cells following temperature and pH alterations. Sales et al. (2017) reported similar morphological changes such as hypertrophy of the respiratory epithelium, lamellar fusion, lifting epithelium and aneurysm on armored catfish (*Hypostamus francisci*) exposed to sewage pollution from industrial effluents. These fish were able to recover within a 30 days recovery period. Such a decrease of the severity of gill alterations within the recovery period was also found in carp exposed to copper under chronic conditions (Karan et al., 1998) and in streaked prochilod in acute condition (Cerqueira and Fernandes, 2002). Thus, we hypothesize that Hoven's carp exposed to an interaction of high temperature and low pH for 20 days with similar symptoms will be able to recover as there were no observed regressive changes that might cause cell damage and organ malfunction such as necrosis or apoptosis.

CONCLUSIONS

Hoven's carp showed high sensitivity towards high temperatures as well as to acidic conditions. The significant changes in the gill filament structure indicated a physiological remodeling strategy with complementary lesions. Hoven's carp gills may be able to recover to their original state when the environmental stress ends as most of the morphological changes were reversible. Nevertheless, if the exposure stress is persistent for long periods, it may result in severe alterations, which deserves further investigation.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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AUTHOR CONTRIBUTIONS

HJL and GDB designed and supervised the project and wrote the manuscript. SM and RAZ performed the experiments and responsible for technical detail during culture period and histology laboratory. SM analyzed the data and wrote the manuscript. SR assisted experimentation and data analysis. SR, KW, HTN and GDB provided inputs and guided writing with constructive suggestions. LQ and YC helped in early experiment design and financial support in laboratory

analysis. MAG provided additional financial support for experiment and publication fee. All authors provided critical feedback and commented to improve the manuscript quality.

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List of Figure Captions

Figure 1. Morphological alterations in the gills of Hoven's carp under ambient temperature in neutral pH (28N) stained with Hematoxylin and Eosin. A) middle region of gill filament under 200x magnification with 25µm scale: Primary lamellae (PL), secondary lamellae (SL), basal epithelial thickness (BET), secondary lamellae length (SLL), secondary lamellae base length (SLBL), secondary lamellae width (SLW), secondary lamellae perimeter (SLP) and interlamellar distance (ID); B) primary lamellae: undifferentiated epithelial cell (UEC) and ionocyte (CC); C) secondary lamellae: red blood cell (RBC) along the capillary and pillar cell (PC) supporting the blood capillary. Figure 1(B-C) was taken under 600x magnification with 25µm scale.

Figure 2. Morphological alterations in the gills of Hoven's carp under high temperature in neutral pH (32N) stained with Hematoxylin and Eosin. A) middle region of gill under 100x magnification with 50µm scale; B) curling of secondary lamellae (long arrow); C) early stage of aneurysm mainly at the tip of secondary lamellae (arrowhead); D) oedema formation mainly on primary gill lamellae (short arrow), hypertrophy of epithelial cell (white arrowhead) and ionocyte (single asterisk in circle). Figure 2(B-D) was taken under 600x magnification with 25µm scale.

Figure 3. Morphological alterations in the gills of Hoven's carp under ambient temperature in acidic pH (28A) stained with Hematoxylin and Eosin. A) a middle region of gill filament under 100x magnification with 50µm scale; B and D) aneurysm at the tip of secondary lamellae (arrowhead); C and E) epithelia cell hyperplasia caused fusion of several lamellae (double

asterisk); F) ionocyte (single asterisk in circle). Figure 3 (B-F) was taken under 600x magnification with 25 and 10µm scale.

Figure 4. Morphological alterations in the gills of Hoven's carp under high temperature in acidic pH (32A) stained with Hematoxylin and Eosin. A) middle region of gill filament under 100x magnification with 50µm scale; B) early stage of oedema formed on primary lamellae and along secondary lamellae (short arrow), mild lifting of respiratory epithelial (long arrow); C and D) aneurysm at the tip of secondary lamellae (arrowhead); D) ionocyte (single asterisk in circle). Figure 4 (B-D) was taken under 600x magnification with 25µm scale.

List of Tables

Table 1. The classifications of the reaction pattern, gill alterations and to clarify the important factor of gill structural changes.

Reaction patterns	Gill alterations	Importance factor
Rp1: Circulatory disturbance	Hemorrhage/aneurysm	I
	Intercellular oedema	I
	Epithelial lifting	I
Rp2: Regressive changes	Necrosis	III
	Atrophy	II
Rp3: Progressive changes	Hypertrophy	I
	Hyperplasia	II
	Lamellar fusion	II
	Lamellar curling	II

*Classification based on Bernett et al. (1999).

Table 2. The morphometric measurement of gill filament structure modification exposed to combination temperature and low pH impacts.

Group	Primary lamellar, µm	Secondary lamellar, µm					
	BET	SLL	SLP	SLW	ID	SLBL	PAGE
28N	25.24±0.62 [a]	74.87±2.75 [a]	153.00±5.01 [a]	6.20±0.14 [a]	20.92±0.65 [a]	14.63±0.70 [a]	74.29±0.63 [a]
32N	12.15±0.77 [b]	101.87±9.04 [b]	207.63±18.39 [b]	4.28±0.10 [b]	21.21±0.49 [a]	10.50±0.63 [b]	90.05±0.93 [b]
28A	20.23±1.33 [c]	91.03±6.38 [ab]	191.34±9.97 [ab]	6.70±0.19 [a]	18.41±0.89 [b]	17.16±0.90 [ac]	81.25±1.95 [c]
32A	14.85±0.75 [b]	96.89±6.98 [ab]	203.01±14.07 [ab]	5.10±0.12 [c]	20.35±0.33 [ab]	18.20±1.01 [c]	86.96±1.09 [b]

Note: the measurement of primary and secondary lamellar were taken not in aneurysm, proliferation or fusion area. Basal epithelial thickness (BET), secondary lamellar length (SLL), secondary lamellar perimeter (SLP), secondary lamellae base length (SLBL), interlamellar distance (ID), secondary lamellar width (SLW) and proportion available for gas exchange (PAGE). Different letters as superscripts indicate that mean values between the exposure groups differ significantly ($P<0.05$) (n=10).

Table 3. Significance levels of the impact of temperature, pH and their interaction in Hoven's carp gill morphology.

	Temperature		pH		Temperature * pH	
	F-value	*P-value*	*F-value*	*P-value*	*F-value*	*P-value*
BET	107.361	0.000	1.692	0.203	18.747	0.000
SLL	6.004	0.020	0.700	0.409	2.490	0.125
SLP	6.349	0.017	1.641	0.209	2.665	0.112
SLBL	3.474	0.071	37.990	0.000	9.710	0.004
ID	3.217	0.084	7.338	0.011	1.771	0.194
SLW	170.988	0.000	24.053	0.000	1.296	0.264
PAGE	77.964	0.000	2.541	0.121	17.108	0.000

Basal epithelial thickness (BET), secondary lamellar length (SLL), secondary lamellar perimeter (SLP), secondary lamellae base length (SLBL), interlamellar distance (ID), secondary lamellar width (SLW) and proportion available for gas exchange (PAGE).

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22 **Table 4.** Histopathologically index in gill of Hoven's carp exposed to combination temperature

23 and low pH impacts.

Reaction pattern	Gill alteration	Importance factor	Group 28N	32N	28A	32A
Rp1	Aneurysm	I	0	2	2	2
	Intercellular oedema	I	0	4	0	2
	Epithelial lifting	I	0	0	0	2
Rp3	Hypertrophy	I	0	6	0	4
	Epithelial Hyperplasia	II	0	0	2	0
	Lamellar fusion	II	0	0	2	0
	Lamellar curling	II	0	2	0	0

24 Note: Score value of lesion presence: (0) unchanged; (2) mild; (4) moderate; (6) severe.

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Table 5. Total gill pathological index of Hoven's carp exposed to combination temperature and low pH impacts.

Group	Rp1	Rp3	I_{gill}
32N	6.40±0.27 [a]	11.60±0.58 [a,*]	18.00±0.60 [a]
28A	1.75±0.25 [b]	8.00±0.00 [b,*]	9.75±0.25 [b]
32A	8.40±0.72 [c]	3.60±0.27 [c,*]	12.00±0.78 [c]

Note: Total pathological index (I_{gill}) for ambient temperature in neutral pH (28N) was excluded as they exhibited normal branchial morphology. Asterisk (*) indicate significant different of Rp1 and Rp3 within the exposure group ($P<0.05$). Different letters as superscripts indicate that mean values among the exposure groups differ significantly ($P<0.05$) (n=10).

Figure 1

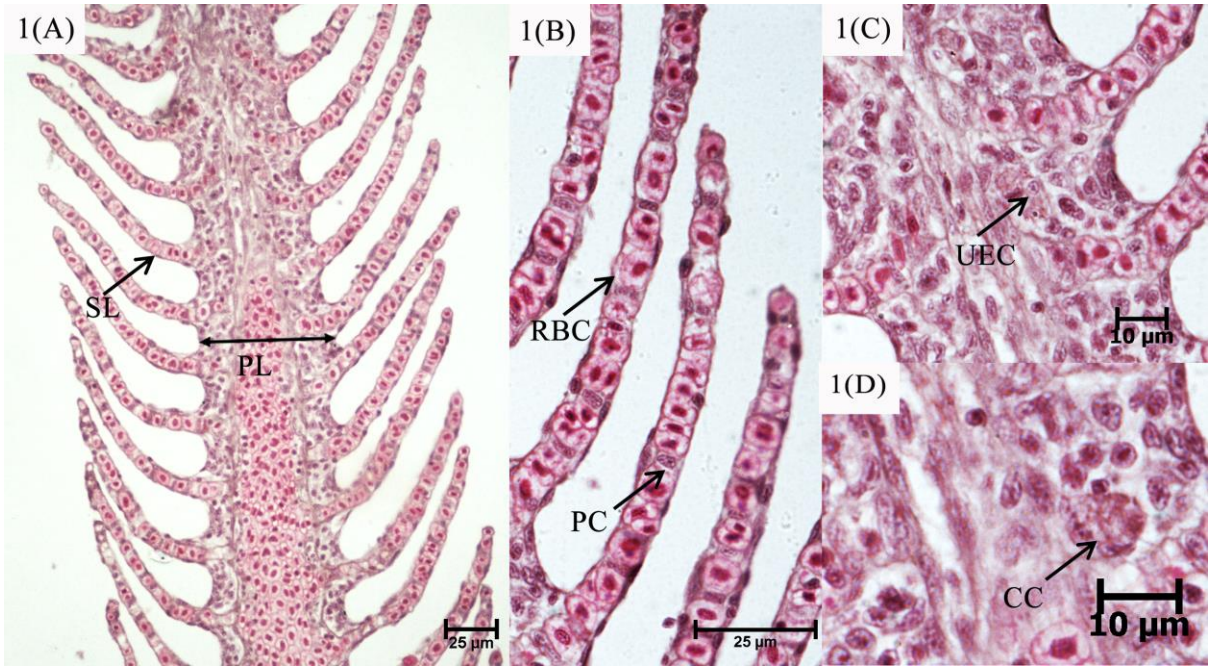

Figure 2

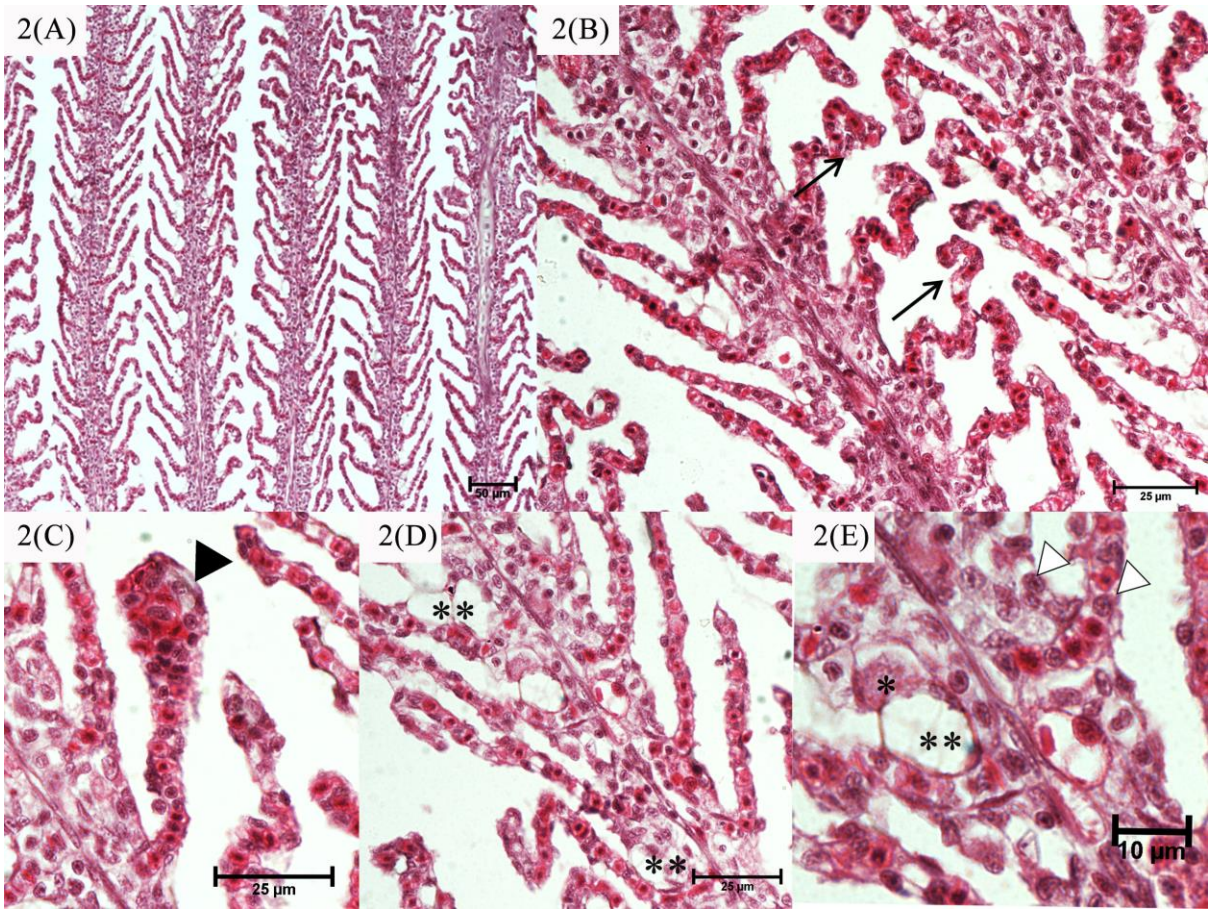

Figure 3

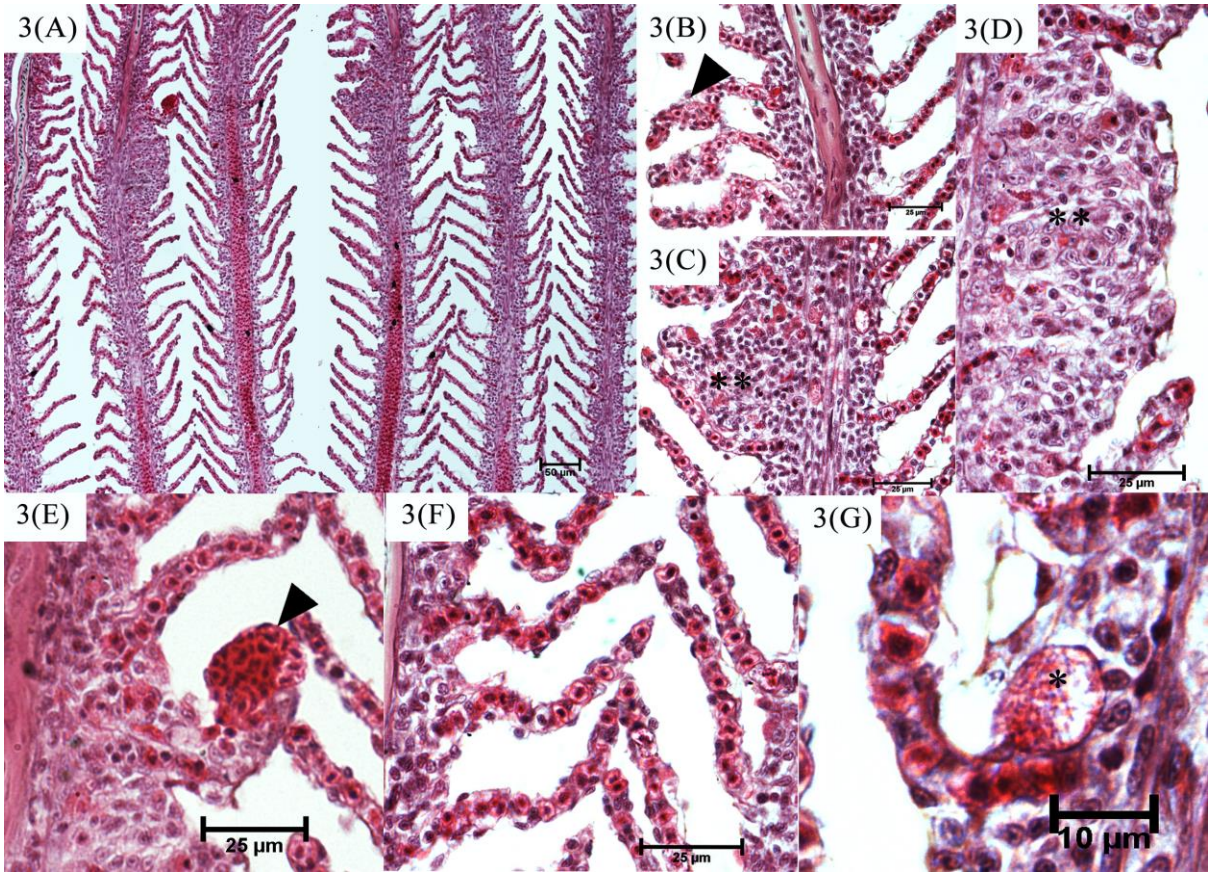

Figure 4