東海大學生命科學系碩士論文

低氧環境對水生呼吸空氣魚類接吻鱸其離子

調節、肝醣代謝及抗氧化能力之影響

The effects of hypoxia on ionoregulation, glycogen metabolism

and antioxidant defenses in the aquatic air-breathing fish,

Helostoma temminckii

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摘要

近來由於全球暖化造成海洋溫度增加，以及人為排放含氮廢物等使水體優養化，因而降低水體溶氧量，持續的低氧會危害到生活於水中的生物。為使能量達到供需平衡，細胞從有氧呼吸轉變成無氧呼吸，行肝醣分解反應（glycogenolysis）以降解肝醣做為緊急燃料。而為減少 ATP 消耗，鈉鉀幫浦（sodium-potassium adenosine triphosphatase，Na^+, K^+-ATPase，NKA）的表現會被抑制。在肝醣分解的過程中，肝醣磷酸化酵素（glycogen phosphorylase，GP）將肝臟與肌肉中的肝醣分解為葡萄糖-1-磷酸（glucose-1-phosphate）的單糖，進而使其繼續進行的糖解作用（glycolysis）。先前研究顯示，魚類鰓部離子調節細胞旁具有富含肝醣細胞（glycogen-rich cells，GR cells），並在面臨環境壓力時進行分解以提供 ATP 予鈉鉀幫浦。此外，生物在低氧時會產生大量自由基，若抗氧化能力不佳，則自由基的產生會使細胞損傷，因此生物如何調節其生理以適應低氧是十分重要的課題。本實驗以攀鱸亞目的接吻鱸（*Helostoma temminckii*）為實驗動物，其為水生型呼吸空氣魚類，具有特化輔助呼吸空氣的器官－迷器，可幫助鰓部進行氣體交換，利於在溶氧極低的環境中生存。在先前的實驗中，同一亞目的電光麗麗（*Trichogaster lalius*）於低氧處理第三天時並無觀察到鈉鉀幫浦的抑制，而接吻鱸的鰓部型態與電光麗麗相似，是否也有相似的反應則未知。為瞭解在三天期間呼吸空氣魚類是否具有肝醣分解而使鈉鉀幫浦獲得能量，則需對此期間的生理變化進一步研究。本實驗假說為，接吻鱸鰓部鈉鉀幫浦未被抑制是否與富含肝醣細胞中的肝醣磷酸化酶分解肝醣，進而提供能量有關；其抗氧化能力亦佳，能夠抵抗低氧時自由基的傷害。實驗結果顯示，接吻鱸在低氧時會增加呼吸空氣頻率。GP 蛋白質表現在低氧期間有上升，但 NKA 表現則未隨之改變；而鰓部及肝臟的肝醣含量在低氧下均有降解。在抗氧化能力方面，鰓部超氧化物歧化酶（superoxide dismutase，SOD）活性在低氧下較回復組高，穀胱甘肽過氧化酵素(glutathione peroxidase，GPx)在低氧及回復組均提升，而穀胱甘肽轉移酶（glutathione-s-transferase，GST）則在低氧及回復組均上升。此外，在肝臟的部分，超氧化物歧化酶在低氧處理時上升，觸酶（catalase，CAT）在低氧下較回復組高，穀胱甘

肽還原酶（glutathione reductase，GSR）在低氧時也有上升。由實驗結果可知接吻鱸可調節其行為、生化及生理反應以存活於低氧環境。

Abstract

Recently, the rising in water temperature and the eutrophication caused by global warming, and anthropogenic discharge of the nitrogenous wastes, lead to a decrease in aquatic oxygen solubility. Serious consequences for aquatic life could be expected if the hypoxic condition prolongs for a period of time. To balance the O_2 and metabolism demands, organisms may change from aerobic to anaerobic respiration by utilizing glycogen as the emergency fuel in glycogenolysis, and the function of sodium-potassium adenosine triphosphatase (Na^+/K^+-ATPase, NKA) may be depressed to preserve energy consumption. In the process of glycogenolysis, glycogen phosphorylase (GP) degrades glycogen in muscle and liver into glucose-1-phosphate for glycolysis. According to previous studies, the glycogen-rich cells (GR cells) in fish gills located right next to mitochondria-rich cells (MR cells). The degrading of glycogen in GR cells could, therefore, cope with high ATP demand from NKA in MR cells when fish is under stress. In addition to the metabolic suppression, organisms produce free radicals harmful to cells in the hypoxic and recovery conditions. Therefore, how organisms adapt to hypoxia is an important issue for survival. The aquatic air-breathing anabantoid fish *Helostoma temminckii*, with the accessory air-breathing organ labyrinth organ connected with the gills, can live in the hypoxic environment. From the previous studies, it is known that no inhibition of NKA protein abundance and activity in hypoxia was observed within the first three days of the experiment in the other air-breathing fish *Trichogaster lalius* which has similar gill morphology with *H. temminckii*. In the present study, it is hypothesized that GP in GR cells degrades glycogen to generate ATP so that NKA in the gills of *H. temminckii* will not be inhibited. *H. temminckii* has better antioxidant ability and this will help to prevent from free radical damages in hypoxia and recovery in normoxia. The result indicates that the aquatic air-breathing *H. temminckii* increased the air-breathing frequency under hypoxia. The protein abundance of GP increased during hypoxic treatment,

but expression of NKA did not change accordingly. Glycogen contents degraded during hypoxic condition in the gills and liver. For the antioxidant mechanism in the gills, the superoxide dismutase (SOD) activity in recovery group was higher than that in the hypoxic group and the glutathione peroxidase (GPx) increased under hypoxic and recovery groups. Moreover, glutathione-s-transferase (GST) activity increased in the recovery group. In the liver, the SOD increased under hypoxia and, catalase (CAT) activity in recovery group was higher than that in the hypoxic group. Furthermore, glutathione reductase (GSR) activity increased in the recovery group. In conclusion, *H. temminckii* regulates its behavioral, biochemical and physiological conditions for surviving under stress condition.

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Introduction

Oxygen is one of the most indispensable ecological factors for all aerobic life. In aquatic ecosystem, organisms are under the threat of hypoxia or anoxia due to the low solubility of oxygen to aquatic environments. Since the developments of agriculture and industry, nutrients and nitrogenous wastes discharged by human activities, result in eutrophication and decreasing in oxygen solubility. Recently, the global climate leads to a rise in water temperature and stratification, thereby reduces dissolved oxygen and supply (Wu, 2002; Rabalais et al., 2009). In tropical and subtropical areas, the freshwater organisms undergo fluctuation of oxygen concentration during dry season. The low oxygen condition of native niches combined with global climate change and anthropogenic effects will accelerate the deterioration of water quality for aquatic environments, especially shallow ditches and river banks.

Fish is the major species in the aquatic system, and its adaptation to prolonged hypoxia is under great concern. Upon encountering hypoxic condition, many fishes adjust their behavior first by escaping from the unsuitable environment (Urbina et al., 2011) and some increase the air-surface respiration or air-breathing frequency (Takasusuki et al., 1998; Timmernan and Chapman, 2004; Gonzales et al., 2006; McNeil et al., 2007). For species that survive under extended periods of hypoxia, fishes adopt morphological modification to exchange more oxygen. For example, the air-breathing fishes have accessory air-breathing organs, such as labyrinth organ, skin, gas bladders, lungs, digestive tracts, and structures derived from buccal, pharyngeal, and branchial cavities (Graham, 1997), and some species remodel the gill surface area to increase the oxygen uptake (Chapman et al., 2000; Sollid et al., 2003, 2005; Matery et al., 2008). However, when fish cannot escape from the hypoxic environment, they tend to undergo metabolic adjustments by reducing energy consumption. This can be achieved through metabolic suppression and ion channel arrest (Bickler and Buck, 2007). Since ATP production is limited under

hypoxia, energy conservation is an important issue for organisms (Bickler and Buck, 2007). Under oxygen-limited condition, ATP is reserved by decreasing the plasma membrane permeability known as "channel arrest" (Hochachka, 1986; Boutilier et al., 2000; Boutilier, 2001). The ion-pump of Na^+, K^+-ATPase, a ubiquitous membrane-bound enzyme, not only provides driving force for ion transport but maintains the membrane potential homeostasis (Marshall et al., 2002; Evans, 2005). NKA consumes approximately 20-80% of cell's resting metabolic rate (Boutilier, 2001); therefore, NKA reduction could be an indicator as channel arrest. NKA activity was depressed in several tissues during anoxic or hypoxic exposure in various species (e.g. *liver* Buck and Hochachka, 1993; Bogdanova et al., 2005; *brain* Hylland et al., 1997; Wilkie et al., 2008; *muscle* Donohoe et al., 2000). In teleost, gills and kidney are the major osmoregulatory organs, and NKA is located mainly in the specialized ionocyte called mitochondria -rich cells (MR cells) of gill epithelia (Lee et al., 1998; Hwang et al., 1999) and epithelia of kidney tubules (Ura et al., 1996). Recently, the studies on the hypoxia-tolerant freshwater fish Amazonian cichlid (*Astronotus ocellattus*) indicated a decrease in energy expenditure by reducing the NKA activity of gill and kidney and transcellular permeability of gill in response to hypoxia (Richards et al., 2007; Wood et al., 2009). Similarly, while the hypoxia-tolerant seawater fish European flounder (*Platichthys fesus*) was acclimated under hypoxia, its NKA activity and mRNA expression diminished significantly (Lundgreen, 2008). On the contrary, the branchial activity of hypoxia-sensitive rainbow trout (*Oncorhynchus mykiss*) did not change under acute hypoxia, suggesting its difference in regulatory mechanisms with that of the hypoxia-tolerant species (Iftikar et al., 2010).

At the metabolic suppression level, fish reduce metabolism and carry out anaerobic respiration to yield ATP as energy supply during extended hypoxia (Hochachka, 1986; Guppy and Withers, 1999; Brickler and Buck, 2007). Glucose is from glycogen by glycogenolysis; therefore, the utilization of glycogen is important for fish in hypoxia.

Glycogen, a very large, branched polymer of glucose, is a readily mobilized storage resource and serves as an emergency fuel in the highly energy-dependent organs, such as brain, liver and muscle. During glycogenolysis, glycogen degrades to glucose-1-phosphate by glycogen phosphorylase (GP), the rate-limiting step before converted to glucose-6-phosphate which, in turn, enters to one of the three metabolic pathways including (1) becoming the initial substrate for glycolysis, (2) entering the pentose phosphate pathway to yield NADPH and ribose; and (3) converting into free glucose in the liver for releasing into the bloodstream (Berg et al., 2002). In addition to what was found in muscle, liver and brain, glycogen deposits in GR cells located next to the mitochondrial-rich cells (MR cells) in the Mozambique tilapia's (*Oreochromis mossambicus*) gills (Tseng et al., 2007). Tilapia gill glycogen phosphorylase (tGPGG) in the GR cells degrades glycogen and releases metabolites. In the study of Chang et al. (2007), when tilapias were transferred from fresh water to 25‰ seawater, both GP activity and relative protein expression in gills were up-regulated, and glycogen content was depleted in both gills and liver. Simultaneously, NKA activity exhibited a significant increase immediately after tilapias were transferred to 25‰ seawater. These results implied that GR cells may involve in providing the energy resources for osmoregulation under stressful condition, and energy transport can be partitioned into local (gill) and central (liver) (Tseng et al., 2008; Lin et al., 2011).

When living in environments with fluctuating oxygen concentrations, organisms not only have to cope with the metabolic adjustment under hypoxia but also the effects of re-introducing oxygen (Storey, 1996; Hermes-Lima et al., 1998). Mitochondria are the major site for oxidative phosphorylation to generate ATP and some electrons leakage from electron transport chain converted oxygen into free radicals which include superoxide radical ($O_2^{-\cdot}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^{\cdot}$) (Hermes-Lima et al., 1998; Hermes-Lima et al., 2001). Those oxygen-containing free radicals carry unstable

electrons are called reactive oxygen species (ROS), which can damage biologically important molecules including nucleic acids, proteins, lipids and carbohydrates (Wilhelm Filho, 1996; Hermes-Lima et al., 1998). To face the oxidative stress, for example, imbalance between ROS generation and removal, the organisms undergo antioxidant defenses (Appendix 1) which include enzymatic and non-enzymatic components (Hermes-Lima et al., 1998). The antioxidant enzymes comprise superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), and glutathione-S-transferase (GST). SOD catalyzes the dismutation of the O_2-· into H_2O_2 and O_2 (Appendix 2, reaction 1) and thus forms a crucial part of the cellular antioxidant defense mechanism (Hermes-Lima et al., 2001). Catalase degrades H_2O_2 (Appendix 2, reaction 2) that performs the same function as glutathione peroxidase (Appendix 2, reaction 3), a selenium dependent enzyme which decomposes H_2O_2 by using glutathione as its co-substrate (Hermes-Lima et al., 2001). NADPH-dependent glutathione reductase (GSR) catalyzes oxidize glutathione (GSSG) back into GSH (Appendix 2, reaction 4) which balances the concentration of GSH (Hermes-Lima et al., 2001).
Glutathione-S-transferase also plays a key role in cellular detoxification (Appendix 2, reaction 5). GST detoxifies the intra- and extracellular xenobiotics which attack functional molecules by conjugation of xenobiotics with GSH (Storey, 1996). During the transition between different oxygen concentrations (hypoxia/anoxia-reoxygenation, estivation-arousal and freeze-thaw exposure), the organisms enhance their antioxidant enzymes to overcome oxidative stress (e. g. garter snakes *Thanmnophis sirtalis* (Hermes-Lim and Stroey, 1993), land snails *Otala lactea* (Hermes-Lima and Storey, 1995ab), African lungfish *Protopterus dolloi* (Page et al., 2010)). Likewise, the evidences of induction in antioxidant enzymes also were reported in the hypoxia-tolerant fish goldfish *Carassius auratus* (Lushchak et al., 2001) and the common carp *Cyprinus carpio* (Lushchak et al. 2005). Nevertheless, the above studies focus on the antioxidant defenses

of the organs, such as brain, liver, kidney and muscle, but not that of the gills (Wilhelm Filho, 2007) which are in direct contact with the aquatic environment and responsible for gas exchange, ionoregulation, acid-base regulations, excretion of nitrogenous wastes (Evans, 2005), and elimination of H_2O_2 (Wilhelm Filho et al., 1994). Although the study on *Leiostomus xanthurus* included gill SOD activity under various oxygen concentrations (Cooper et al., 2002), information for the whole antioxidant enzymes in the gills was not known during the periods of hypoxic and reoxygenation exposure.

Air-breathing fish are the kind of fish that possesses the ability to exchange gas directly via the aerial environment, and they are classified into amphibious and aquatic air-breathing fish according to their behavior and habitat (Graham, 1997). Since most of them live in the habitats where O_2 supplies are severely depleted, they develop strategies to cope with hypoxia by having accessory air-breathing organs. All Anabantoidei have the labyrinth apparatus, a bony structure formed by the epibranchial segment of the first gill arch (Graham, 1997). There are 120 species in three families of Anabantoidei, including Anabantidae (33 species), Helostomatidae (1 species) and Osphronemidae (86 species) (Helfman et al., 2009). The Helostomatidae, kissing gourami (*Helostoma temminckii*), is investigated in the present study. *H. temminckii* distributes in Malaysia, Burneo, Sumatera and South Asia. It is found in brackish water (estuaries/lagoons/brackish seas/mangroves) and fresh water (rivers/streams/lakes/ponds) (Fishbase, http://www.fishbase.org/) and reached a maximum length of 30 cm (Graham, 1997). According to the previous study from our laboratory, no inhibition of NKA protein expression and activity was observed within the first three days of acclimation to hypoxia in *Trichogaster lalius*, a species with similar gill morphology as *H. temminckii*. The result of no inhibition of NKA in this hypoxia-tolerant *T. lalius* is similar to what was found in the hypoxia-sensitive rainbow trout. In the antioxidant defense, there are few studies about hypoxia-tolerant air-breathing fishes. Therefore, in the present study, it is hypothesized that (1) GR cells in the gills of *H.*

temminckii will undergo glycogenolysis to maintain NKA activity in the nearby MR cells (Appendix 2) and the glycogen in the liver will be degraded into glucose and transported to gill; (2) the antioxidant enzymes activities of *H. temminckii* will increase for resisting the ROS damage.

Materials and Methods

Experimental Animals

The kissing gouramis (*Helostoma temminckii*), 3.23 ± 0.62 cm in body length, were purchased from a commercial fish supplier and held in the animal room for at least a week prior to use in any experiment. Fish were kept in 140 l plastic tank (75 × 54 × 40 cm) local tap water (fresh water) with aerated and circulated system at 27± 1°C under a 12 h: 12 h light-dark photoperiod. One third of the water was replaced every week. Fish were fed commercial fish food pellets (NOVO Bits, JBL, Germany) *ad libitum* twice daily. One day before experiment, fish were transferred into glass tank (35 × 20 × 22.5 cm^3) filled with aerated local tap water to a height of 20 cm (Normoxia). Fish were not fed one day before and during experiment.

Experimental Design and Sampling

Normoxia was aerated by pumping air and hypoxia maintained by gassing with N_2. After one day acclimated in normoxia, fish were transferred to the following two groups: 1) control (normoxia) and 2) hypoxia (0.81±0.17 mg/l). The experimental tanks were glass tanks (35 × 20 × 22.5 cm^3) filled with freshwater at 20 cm height and temperature controlled at 27± 1°C. The dissolved oxygen (DO) (Thermo, Orion model 810, UK) and pH (Jenco, pH vision 6071, HK) of the tanks were monitored at 8.13±0.52 mg/l and 7.52±0.40. Fish were sampled at 0, 3, 6, 12, 24 and 72 h under normoxia and hypoxia. In some experiments, hypoxic fish were returned to normoxic water for 1 and 12 h. The sampling time is different among experiments, and is listed in Table 1. The time course experiment was repeated at least 8 times. The body length of the fish and water quality under different treatment conditions were listed in Table 1.

Air-breathing Frequency

After acclimated in the normoxic water, the fish was transferred to hypoxic tank for 72 h. Video cameras were to record the air-breathing behavior which was defined as when the fish swam up to the border of water and air and gulped air. The behavior in normoxic condition prior to the transferal to hypoxia was the control. To eliminate the possible handling effect, normoxic condition was recorded 3 h after transferal. The hypoxic condition was recorded for one hour each at 3, 6, 12, 24, 72 h, and the rate of breathing was by determining the numbers of air-breathing events divided by the length of the recording periods.

Protein Extraction

Fish were sacrificed by severing spinal cord on ice, and the gills, liver, and kidney were excised rapidly. The tissues were immersed in PBS (136.9 mM NaCl, 2.68 mM KCl, 10.15 mM $Na_2HPO_4 \cdot 2H_2O$, and 1.76 mM KH_2PO_4, pH=7.4) and the blood clots removed with forceps. Each tissue was homogenized (Ultrasonic Processor, SONICS, USA) in 200 μl homogenization solution (100 mM imidazole, 5 mM Na_2EDTA, 200 mM sucrose and 0.1% sodium deoxycholate) containing a mixture of proteinase inhibitor (3.31 mM antipain, 2.16 mM leupeptin and 1.92 M benzamidine) in an aprotinin saline solution (5-10 trypsin inhibitor units per ml, Sigma, MO, USA) in a volume ratio of 200:1 at a pH of 7.6. Homogenate was centrifuged at 20,160 *g* for 30 minutes at 4°C (EBR12R, Hettich, Germany) to obtain supernatant. 2 μl of supernatant was diluted to 200 μl with deionized water, and an aliquot of 100 μl mixture was further diluted to 800 μl with deionized water. The diluted protein solution was added 200 μl protein assay (Bio-Rad Laboratories, Hercules, CA, USA). The extracted protein concentration was measured by a spectrophotometer (U-2001, Hitachi, Japan) at a wavelength of 595 nm. The bovine serum albumin diluted with deionized water was treated as the standard, and the mode of dilution

was identical with samples. The standard concentration was 0, 2, 4 and 8 μg/μl.

Antibodies

The antibodies were NKA α-subunits (NKA, 1:5000, α-5 monoclonal antibody; from chicken, DSHB, USA), GPBB (1:3000, monoclonal antibody, from mouse, Biotrend Chemikalien GmbH, Cologne, Germany), and β-actin (1:10000, monoclonal antibody; from mouse, Chemicon, USA). The secondary antibody is peroxidase-affinipure goat-anti-mouse IgG (1:10000, Jackson Immunoresearch Laboratories, West Grove, PA, USA) to detect the above primary antibody. All antibodies were used in the following study.

Western Blotting Analysis

The protein samples (total protein: gill 25 μg, kidney 25 μg and liver 50 μg) were added with 4× electrophoresis sample-loading buffer (20 mM Tris-HCl, pH 6.8, 8% sodium dodecyl sulfate (SDS), 10% β-mercaptoethanol, 40% glycerol and 0.4% bromophenol blue). Protein samples were incubated at 37°C for 15 minutes for NKA and 95°C for 10 minutes for GP. The denatured proteins were subjected to 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) at 110 V for 2.5 h. After transferred to polyvinylidene difluoride membranes, the blots were blocked in 5% nonfat milk in PBST buffer (NaCl 136.9 mM, KCl 2.68 mM, $Na_2HPO_4 \cdot 2H_2O$ 6.39 mM, KH2PO4 1.76 mM and 0.5% Tween 20, pH7.4) for 1 h at room temperature, and then washed 3 times with PBST buffer for 30 minutes. Blots were incubated with a monoclonal primary antibody (NKA, 1:5000; GP, 1:3000 and Actin, 1:10000) overnight at 4°C, respectively. The blots were washed 5 times with PBST buffer for 1 h, and then were reacted for another 1 h with peroxidase-affinipure goat anti-mouse IgG. After washed with PBST buffer 5 times for 1 h, immunoreactive proteins were detected using western blot

chemiluminescence reagent plus system (NEN Life Science) to identify the concerned proteins. The signals were captured and photographed by an Intelligent Dark Box II with Fujifilm LAS-1000 digital camera and the relative protein abundance was estimated and analyzed using an Image Gauge 4.0 (Fujifilm). The relative protein abundance was the target protein divided by β-actin (internal control) in each set.

Na^+, K^+-ATPase Activity Assay

Aliquots of the suspension of gill and kidney homogenates prepared as described above were used for determination of enzyme activities. NKA activity was assayed by adding the supernatant to the reaction medium (containing imidazole-HCl buffer 142.85 mM pH 7.6, NaCl 178.5 mM, $MgCl_2$ 10.71 mM, and KCl 107.14 mM). Each sample was assayed in triplicate. The inorganic phosphate concentration was modified according to the method of Peterson (1978) using microplate. Enzyme-specific activity was defined as differences between the inorganic phosphate liberated in the presence and absence of protein inhibitors. Ouabain was used as inhibitor for NKA. For measuring NKA activity, the inhibitor-free solution (1600 µl) comprised 800 µl of reaction medium, 400 µl of 10 mM ouabain. In each well, 2 µl of sample supernatant was added to 40 µl of mixed inhibitor-free or enzyme-inhibitor solution. Then, 10 µl Na2ATP 30 mM was added and incubated at 37°C for 30 minutes. Using 100 µl of Bonting's color reagent ($FeSO_4$ 176 mM, H_2SO_4 560 mM, and ammonium molybdate tetrahydate 8.1 mM) to react with reaction mixture, the solution was incubated and shaken at 20°C for 30 minutes in the dark. The inorganic phoaphate concentration was determined by spectrophotometer with 690 nm (ELISA, Enzyme Linked ImmunoSorbent Assay; Thermo, USA). Enzyme specific activity was expressed as µmol $Pi \cdot mg^{-1}$ $protein \cdot h^{-1}$.

Preparation of mRNA and cDNA Synthesized from mRNA

In the process of preparing mRNA, isolated gill and liver tissues were homogenized in 1 ml of Trizol reagent (Invitrogen, Carlsbad, CA, USA) and treated according to the manufacturer's protocols. The amount and quality of total RNA were determined by measuring the absorbance at 260 and 280 nm with a spectrophotometer (SSP-3000, Infingen Biotechnology Inc., USA) and analysis using sybersafe-staining (Invitrogen, Carlsbad, CA, USA) gel. For first-strand cDNA synthesis, 1μg of the total RNA was reverse transcribed in a final volume of 20 μl containing 10 mM dNTPs, 10 mM $MgCl_2$ and 2.5 μM oligo$(dT)_{20}$ of SuperScript^{TM} III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instruction. *Escherichia coli* RNase H were added to remove the remnant RNA. The cDNA samples were finally stored at -20 °C prior to the experiments.

Quantitative Real-time PCR

Quantitative real-time PCR (qPCR) was performed with the LightCycler® 1.5 Instrument (Roche Molecular Biochemicals, Mannheim, Germany) using the DNA binding dye SYBR Green I for the detection of PCR products. cDNA was amplified in the reaction mixture (10.1 μl) containing 0.9 μl LightCycler FastStart DNA Master SYBR Green I, 0.5 μM of each primer, and 3 mM of $MgCl_2$ using LightCycler. The thermal profile of the LightCycler was optimized with an initial denaturation of 10 min at 95 °C and 35 amplification cycles with each 10 s at 95 °C, 10 s at 60°C and 10 s at 72 °C. After completion of amplification, a melting curve analysis was performed by heating the product at 95°C, cooling it at 65°C, and then heating it again at 95°C with fluorescence collection at 0.1°C/s intervals. The resulting melting curve was used to establish the specificity of the amplicons produced.

PCR data were analyzed using the LightCycler Software (Version 3.5). A relative

quantification was performed using a serial dilution of cDNA (dilutions for concentrated samples are, 1:10, 1:50, 1:100, 1:500, 1:1000) as external standard in each experiment. Standard curves were generated by the Fit Point Method of the LightCycler Software. The relative amounts of NKA, GP and β-actin (internal control) transcripts for all samples were calculated automatically using a standard curve. Then the mRNA expression level of target genes (NKA and GP) was normalized by reference gene (β-actin). Primers for all genes were designed using Primer 3-Plus and were listed in Table 2.

Glycogen Content

Glycogen content was determined according to the method of Chang et al. (2007). Gill and liver tissue were homogenized in 30% KOH (Union Chemical Works Ltd., Hsinchu, Taiwan) and heated in a boiling water-bath for 30 min. After heating, the tubes were centrifuged at 6000 rpm for 5 min. The supernatants were collected from tubes, and the volume was estimated. The removed supernatants were supplemented with two volumes of 100% ethanol and incubated overnight at 4°C. Glycogen was precipitated by centrifugation at 6000 rpm, 10 min after the addition of two or three drops of Na_2SO_4 (Merck, Darmstadt, Germany). The glycogen pellets were washed with 66% ethanol and centrifuged at 6000 rpm, 10 min. Then the pellets were incubated at 37°C until it completely dried. The glycogen content was analyzed in 0.2% anthrone reagent (Alfa Aesar, Heysham, England) dissolved in 99% H_2SO_4 (Merck, Darmstadt, Germany). The absorbance was determined using ELISA reader with a wavelength of 620 nm.

Antioxidant Ability

Tissues were homogenated in homogenation buffer (20 mM HEPES, pH 7.2, containing 1 m MEDTA, 70 mM sucrose and 1% Triton X-100) and centrifuged at 10,000 *g* for 15 minutes at 4°C. The supernatant was collected for assaying the experiment.

Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GSR) and glutathione s-transferase (GST) activities were determined using the Cayman kit (Cayman Chemical Company, Ann Arbor, MI, USA).

Statistics

All values are presented as means ± S.E.M. For statistical analysis, the data were analyzed using one-way analysis of variance (ANOVA) and Duncan's post hoc test, and the Glycogen content, mRNA and protein expression of GP were analyzed using Dunnett's post hoc test by comparing them with the control group. In addition, control and treatment groups at the same time points were compared with Student's *t*-test. Differences were considered statistically significant where $p<0.05$. All statistical analyses were conducted using SAS 9.1 for Windows (SAS Institute, Cary, NC, USA).

Results

Air-breathing Frequency

The frequency of the air-breathing (Fig. 1) was significantly higher in the hypoxic periods at hours 3, 6, 12, 48 and 72 (one-way ANOVA, $F_{5, 35}$= 11.91, p<0.0001; Duncan's post hoc test, p<0.05) than in the normoxic condition. The frequency of air-breathing under the hypoxia elevated at 3-12 h before it declined gradually.

Expression of Ionoregulatory Protein

a. NKA Protein Expression in Gill and Kidney

There were two NKA immunoreactive bands at approximately 95 kDa in the gill of *H. temminckii* acclimated to control and hypoxic groups. Since the expression patterns of the two bands in the different time course were similar, the two bands were included in the analysis at the same time. The relative protein abundance of NKA (Fig. 2 A) were not significantly different between control and hypoxic groups (one-way ANOVA, $F_{5, 47}$=0.99, p=0.44) in the gill. The level of NKA protein expression showed an up-regulation at 3-6 h followed by a down-regulation at 12-24 h, and finally regained at 72 h.

There was only one NKA immunoreactive band in the kidney, the molecular mass of NKA in kidney is also 95 kDa. The relative protein abundance of NKA (Fig. 2 B) were not different among control and hypoxic groups (one-way ANOVA, $F_{5, 47}$=2.23, p=0.07). The level of NKA protein expression showed up-regulation instantly after transfer to hypoxia, and the highest expression was observed at 24 h (Duncan's post hoc test, p<0.05) before a slight down-regulation at 72 h.

b. NKA Enzyme Activity in Gill and Kidney

The specific activity of NKA in gill (Fig. 3A) was not different (one-way ANOVA,

$F_{5, 47}$=0.95, *p*=0.46) among control and hypoxic groups. The trend of activity increased at 3 h, and recovered back to the same level as the control. Then, the activity was up-regulated at 24 h.

In the kidney, the specific activity of NKA (Fig. 3B) did not differ significantly (one-way ANOVA, $F_{5, 47}$=0.26, *p*=0.93) among control and hypoxic groups. The level of activity elevated at 3-6 h, and regained to the level near the control.

c. NKA mRNA Expression in Gill

The NKA α-subunit mRNA expression of the gill (Fig. 4) had no difference among control and hypoxic groups (one-way ANOVA, $F_{3, 31}$=1.30, *p*=0.29). The level of mRNA expression rose between 3 and 12 hr.

Glycogenolysis

a. GP Protein Expression in Gill and Liver

There was a single GP immunoreactive band corresponding to a molecular mass of about 97 kDa. In the gill, the relative protein abundance of GP (Fig. 5A) were different (one-way ANOVA, $F_{10, 87}$=2.88, *p*=0.0042) among control and hypoxic groups. The trend of GP expression increased both under normoxia and hypoxia, and the hypoxic group at 72 h was significantly higher (Dunnett's post hoc test, $p<0.05$) than control group.

GP relative protein abundance of liver (Fig. 5B) showed no difference (one-way ANOVA, $F_{10, 87}$=1.41, *p*=0.19) among control and hypoxic groups, although the expression under hypoxia tended to be higher.

b. GP mRNA Expression in Gill and Liver

Gill GP mRNA level (Fig. 6A) was not significantly different (one-way ANOVA, $F_{6, 55}$=1.29, *p*=0.28) among control and hypoxic groups; however, there was a trend that the

level of mRNA expression increased during hypoxia.

In the liver, GP mRNA level (Fig. 6B) was not significantly different (one-way ANOVA, $F_{6, 55}$=1.18, p=0.33) among control and hypoxic groups, but the expression of hypoxic group was elevated in the acclimated period.

c. Glycogen Content in Gill and Liver

There was a difference between control and hypoxic groups at 12, 24 and 72h (Fig. 7A) (one-way ANOVA, $F_{10, 109}$=2.54, p=0.01, Dunnett's post hoc test, p<0.05). Compared to the control group at the same sampled time, glycogen levels in the hypoxic treatment decreased in the gill at 24 h (Student's t-test, p=0.04) and 72 h (Student's t-test, p=0.04).

The glycogen levels in the liver were significantly different (Fig. 7B) between control and hypoxic group at 6 and 72 h (one-way ANOVA, $F_{10, 109}$=5.71, p<0.0001, Dunnett's post hoc test, p<0.05). Compared to the control group at the same sampled time, glycogen levels in the hypoxic group decreased in the liver at 12 h (Student's t-test, p=0.03) and 72 h (Student's t-test, p<0.0001).

Antioxidant Ability

a. SOD Activity in Gill and Liver

The SOD activity was different among control, hypoxia and recovery groups (one-way ANOVA, $F_{10, 109}$=2.54, p=0.0018, Duncan's post hoc test, p<0.05) in the gill (Fig. 8 A). Compared to that of control group, the activity of hypoxic groups was lower than recovery groups, and the activity of recovery group at 1 h was higher than control and hypoxic groups.

The SOD activity was not different among control, hypoxia and recovery groups (one-way ANOVA, $F_{10, 109}$=1.59, p=0.1845) in the liver (Fig. 8 A); however, the activity of hypoxic groups and recovery groups had a trend that increased at all of time points.

b. CAT Activity in Gill and Liver

The CAT activity of the gill was not different among experimental groups (Fig. 9 A) (one-way ANOVA, $F_{5, 47}$=1.30, p=0.28).

In the liver, the activity showed a difference among hypoxic groups and recovery groups (Fig. 9 B) (one-way ANOVA, $F_{5, 47}$=3.12, p=0.02). The hypoxic groups at 3 and 12 h were significantly different from recovery groups, and the recovery groups were higher than hypoxic groups (one-way ANOVA, Duncan's post hoc test, p<0.05).

c. GPx Activity in Gill and Liver

The GPx activity of the gill (Fig. 10 A) was not different among experimental groups (Fig. 10 A) (one-way ANOVA, $F_{5, 47}$=0.87, p=0.19). However, the control was lower than hypoxic and recovery groups (one-way ANOVA, Duncan's post hoc test, p<0.05).

The GPx activity of the liver (Fig. 10 B) was not different among experimental groups (one-way ANOVA, $F_{5, 47}$=1.57, p=0.1898). However, the activity of hypoxia groups at 3 and 12 h and recovery groups showed the increased pattern.

d. GSR Activity in Gill and Liver

The GSR activity of the gill (Fig. 11 A) was not different among experimental groups (one-way ANOVA, $F_{5, 47}$=0.99, p=0.4353).

The GSR activity of the liver (Fig 11 B) was different among control and hypoxic groups at 3, 12 and 72 h (one-way ANOVA, $F_{5, 47}$=3.55, p=0.0091; Duncan's post hoc test, p<0.05). Although the recovery groups had no significant difference compared with control, they still had an increased pattern.

e. GST Activity in Gill and Liver

The GST activity of the gill (Fig. 12 A) was different among control, hypoxic and

recovery groups (one-way ANOVA, $F_{5, 47}$=2.92, p=0.0236; Duncan's post hoc test, p<0.05). The hypoxic group was elevated at 3h and recovery group was elevated at 12 h.

The GST activity of the liver (Fig. 12 B) was not different between control and recovery group at 12 h (one-way ANOVA, $F_{5, 47}$=2.09, p=0.0850). However, the activity of recovery groups had the trend that increased at 12 h.

Discussion

Air-breathing Behavior under Hypoxia

Fishes utilize different strategies to cope with the oxygen depleted conditions to balance oxygen demands. Behavioral responses are much direct and effective way to avoid hypoxic condition or to transfer oxygen from environment into tissues by air-breathing or aquatic surface respiration (ASR). All Anabantoids are continuous aquatic air-breathers, which breathe air over the whole range of oxygen concentration. Teleost fishes employ air-breathing respiration (Mattias et al., 1998; Takasusuki et al., 1998; Randle and Chapman, 2005; Gonzales et al, 2006; Alton et al., 2007; Huang and Lin, unpublished) or ASR (Kramer, 1983; Timmerman and Chapman, 2004; McNeil and Closs, 2007; Urbina et al., 2011) in response to hypoxia. A rise in the frequency of air-breathing or ASR is an indication of how fishes meet the stress of oxygen loading. *H. temminickii* increased the air-breathing frequency at first followed by a gradual decline (Fig. 1), and this was similar to what was found in sailfin molly (*Poecilia latipinna*) during the 6-wk acclimation in 1.0 mg L^{-1} dissolved oxygen (Timmerman and Chapman, 2004). In addition to the cost of energy and time, the behavior of air-breathing and ASR are also under the possible risk of aerial and aquatic predators (Kramer, 1983; Wolf and Kramer, 1987). Therefore, the physiological and biochemical responses compensating the demand of oxygen become one of the alternative adjustments (Timmerman and Chapman, 2004).

NKA Expression in Gill and Kidney

Since the protein synthesis and ion-motive ATPase are the major energetically costly processes of cells, down-regulating these two ATP-depleted mechanisms are the primary target in order to conserve ATP in response to oxygen deficiency (Boutilier, 2001). NKA consuming about 19-28% of standard metabolic rate takes the greatest priority of ATP

demand (Boutilier, 2001). Previous studies have shown the ion channel arrest in the brain of anoxia-tolerant turtle (*Trachemys scripta*) reduced the NKA activities in 31-34% under anoxia (<0.1 mg L^{-1}, 24 h) (Hylland et al., 1997). Similarly, when goldfish (*Carassius auratus*) were exposed to anoxia, NKA was reduced in the brain by 30-40% for 24 h (Wilkie et al., 2008). In contrast, the other anoxia-tolerant crucian carp (*Carassius carassius*) maintained its NKA activities in the brain (Hylland et al., 1997). The two explanations for the different observation in carp from turtle and goldfish include that: 1) the turtle may become comatose while carp stay active during anoxia (Hylland et al., 1997), and 2) the anoxic period (24 h) was not sufficient to induce NKA activities since crucian carp is more anoxia tolerant than goldfish (Wilkie et al., 2008). Furthermore, exposure to anoxia or severe hypoxia also induced a reduction in Na^+, K^+-ATPase in the hepatocytes of turtles (*Chrysemys picta bellii*) (anoxia) (Buck and Hochachka., 1993) and rainbow trout (*Oncorhynchus mykiss*) (21-0.5 kPa O_2) (Bogdanova et al., 2005). Thus, decreasing in NKA activities in highly energy-consumed organs is necessary for long period of anoxia or hypoxia. In the teleost, the gills, kidneys and intestines are responsible for osmoregulation and consuming about 20-50% of whole-body energy (Febry and Lutz, 1987; Kirschner, 1993; Kirschner, 1995). It is expected that the NKA activities of gill and kidney will decline under anoxia and hypoxia. Suppression of osmoregulation was studied most extensively in hypoxic tolerant fish Amazonian cichlid (*Astronotus ocellatus*). While exposing to acute hypoxic condition (P_{O_2}~10 mmHg), *A. ocellatus* reduced the Na^+ influx and efflux rates and the NKA activities decreased about 60% in gills at 3 h (Wood et al., 2007). In another experiment conducted by same group, a reduction in gill net K^+ fluxes rate and transcellular permeability in Amazonian cichlid during hypoxia were reported (P_{O_2}= 10-20 torr) (Wood et al., 2009). Richards and his colleagues also reported that *A. ocellatus* down-regulated gill and kidney NKA activities by 65% at 4 h exposure to hypoxia (0.37 mg L^{-1}), but maintained the gill NKA protein abundance and elevated the

NKA α1-subunit mRNA level at 8 h (Richards et al., 2007). European flounder (*Platichthys flesus*) was acclimated to different salinity under moderate hypoxia (P_{O_2}=54 mmHg), the down-regulation of NKA activity by 25% and NKA α1-subunit mRNA were found in response to hypoxia instead of salinity (Lundgreen et al., 2008).

In the present study, the channel arrest was not observed in the aquatic air-breathing fish, *H. temminckii*. The mRNA, protein abundance and activity of NKA in gills of *H. temminckii* did not change significantly during the periods of hypoxic exposure (0.8 mg L^{-1}). Similar findings were reported in goldfish acclimated to anoxia; the gill NKA activity did not change during 24 and 48 h (Wilkie et al., 2008). According to Wilkie et al.(2008) no anoxia-induced suppression of NKA was found, because the total gill NKA activity occupies a relative small parts (~1%) of total oxygen consumption in teleost (Morgan and Iwama, 1999). Hence, the channel arrest in the gill will not contribute to save energy. Another possible explanation is that since the goldfish was acclimated to anoxia instead of hypoxia, no gill remodel for the increase of the surface area and, therefore, no changes in gill NKA activity were found (Wilkie et al., 2008). Moreover, in response to hypoxia (P_{O_2}~10 mmHg), the gill NKA activities increased from 1 to 7 days, followed by a rise in the relative mRNA expression thereafter (Mitrovic et al., 2009). It is suggested that the gill remodel for the respiratory surface area also resulted in the increase in the ion diffusion and this, in turn, led to a rise in NKA activity to compensate for the ion loss. There are two conceivable explanations for the no change in NKA expression in *H. temminckii*. First, gill mitochondrial protein synthesis was not depressed in response to acute hypoxia, because the gill is the dominant organ which directly maintains ionic regulation and gas exchange. As stated by Lewis and Driedzic (2010), maintaining the protein synthesis which is ATP-consuming process assures for cellular turnover of gill epithelial and MR cells. Another possible explanation is that glycogen is broken down by GP to supply immediate energy to MR cells for osmoregulation (Tseng et al., 2007; Chang et al., 2007); though, the

NKA expression did not increase with the decomposition of glycogen in gills of *H. temmnickii* like tilapia.

The expression of renal NKA activity in *H. temmnickii* did not change, and the protein level even increased during the periods of hypoxic exposure (0.8 mg L^{-1}).When exposed to hypoxia (P_{O_2}= 10-20 torr), Amazonian cichlid showed a reduction in kidney glomerular filtration rate, urine flow and tubular Na^+ reabsorption rate by 70% (Wood et al., 2009). Hypoxia-sensitive rainbow trout (*Oncorhynchus mykiss*) was found to have the opposite results (Review by Wood et al., 2009). Additionally, hypothermia was found to have a similar effect as hypoxia in metabolic suppression (Hochachka, 1986; Boutilier, 2001). During cold-acclimation (1°C), the renal NKA activity in goldfish did not change. The kidney responded with the cold exposure by decreasing urine flow rate and total sodium loss for maintaining the blood ion concentrations (Paxton and Umminger, 1983); thus, the unchanged renal NKA expression in *H. temmnickii* under hypoxia may have similar effect as the cold-acclimation.

Glycogen Metabolism in Gill and Liver

The changes of GP mRNA and protein abundance and glycogen contents in gill are indications of utilizing glycogen in GR cells for neighbored MR cells. In the present study, NKA expression did not change accordingly, suggesting a constant supply of ATP may follow. Degradation of glycogen is also an indication of glycolytic pathway to compensate for the decrease in ATP production while cells undergo anaerobic respiration (Low et al., 1993). In a previous study on two species of mudskippers (*Periophthalmodon schlosseri* and *Boleophthalmus boddaerti*) acclimated to hypoxia (0.8 µl O_2 $m1^{-1}$, 1 h), these amphibious air-breathing fishes were found to accumulate the lactate and express the lactate dehydrogenase (LDH) in the gill (Low et al., 1993). The detection of lactate and LDH implies that there is the energy transportation in the gill. Works on tilapia

subsequently confirmed that the LDH1 and LDH5 are expressed in MR cells of gill epithelial cells, implying the role of LDH isozymes is in generating ATP during an emergency (Tseng et al., 2008). Moreover, in a recent review by Hwang and his colleagues, the authors indicated that double in situ hybridization/immunocytochemistry provided the evidence for the distribution of the monocarboxylate transporter isoform (MCT4) in both GR cells and ionocytes in zebrafish, and suggested the lactate or pyruvate transports between GR cells and ionocytes (Hwang et al., 2011). *In vitro* studies on HeLa cells also showed that both the mRNA and protein abundance of MCT4, but not MCT1 were induced by hypoxia (1% O_2, 48 h) (Ullah et al., 2006). This *in vitro* study was also confirmed by the *in vivo* studies on zebrafish using cDNA microarray (Ton et al., 2003). When acclimated to long-term hypoxia (20 mmHg, 3 weeks), zebrafish increased the MCT4 gene expression (van der Meer et al., 2005). However, opposite results were also found and it was the MCT1, not MCT4, that was expressed in gills under short-term hypoxia (~30 mmHg, 2-4 days) (Ngan and Wang, 2009). In sum, all studies indicated that glycogen could be degraded and shuttled between cells in order to fulfill the energy requirement under stress conditions. Nevertheless, the air-breathing fishes did not elevate the GP-related gene(s) or protein expression, and, accordingly, it deserves further investigation to confirm whether the carbohydrate translocation is taken place.

While in the stressful condition, organisms convert glycogen to glucose from liver and release to the blood flow before transported to other tissues (Berg et al., 2002). In present study, even though the GP protein abundance increased at 24-72 h and GP mRNA didn't change during hypoxic exposure; liver glycogen level dropped at 6 h after transfer. Our finding supports the idea that the glycogen is a primary glucose energy source in hypoxia (Zhou et al., 2000). The decreasing pattern of glycogen in *H. temminckii* is similar to that of the hypoxia-tolerant common carp, *Cyprinus carpio*. Their liver glycogen levels were depleted by half of the contents in normoxic treatment during 24 h exposure. In the

liver of the anoxia-tolerant crucian carp (*Carassius carassius*), glycogen diminished only during long-term anoxia (<0.1 mg L^{-1}, 8 °C, 17 days) (Nilsson, 1990). However, liver glycogen depleted rapidly after 12 h anoxic (20 °C) but unchanged during hypoxia (P_{O_2} = 17-20 mmHg) in the other anoxia goldfish, *Carassius auratus* (van den Thillart et al., 1980). As for the air-breathing *Channa maculate*, liver glycogen depleted during prolong hypoxia (P_{O_2} = 30 mmHg, 25 °C, 3 days), but not during acute hypoxia (P_{O_2} = 50, 30 and 10 mmHg, 1 h) (Yu and Woo, 1987). In contrast, in their recent work on air-breathing loricariid fish, *Pterygoplichthys anisitsi*, da Cruz and his collaborators (2012) found that liver glycogen concentration decreased under acute hypoxia (without access to atmospheric air, P_{O_2} = 17-20 mmHg, 24±1°C, 3-4 h). The depletion of glycogen reserves are affected by many factors, including at least temperature (Nilsson, 1990), exposure time (Yu and Woo, 1987), exposure conditions (air available or not) (da Cruz et al., 2012). In contrast to our results, there was an increase in the GPase activity in the liver of gulf killifish, *Fundulus grandis* (Martínez et al., 2006) and crucian carp under hypoxia/anoxia (Vornanen and Haverinen, 2011). In *H. temmnickii*, the GPase activity did not change under hypoxia. Perhaps the constitutive expression of GP activity, and the allosteric regulation between GP *a* and GP *b* are sufficient to degrade glycogen while meeting the energy demand (Vornanen and Haverinen, 2011). Some studies revealed the opposite pattern in glycogen contents; for instance, the Amazonian cichlid *Astronotus ocellatus*, liver glycogen increased at 4 h hypoxia (0.37± 0.19 mg L^{-1}) (Richards et al., 2007). In another Amazonian cichlid *Astronotus crassipinnis*, its liver glycogen elevated under hypoxia (0.34 mg L^{-1}) (Chippari-Gomes et al., 2005). Chippari-Gomes and her colleagues proposed that increase in hepatic glycogen is for the following release during hypoxia. On the contrary, the liver glycogen contents in hypoxia-sensitive scaleless fish *Galaxias maculatus* was maintained during 2-6 h hypoxia (Urbina and Glover, 2012). This result is similar to that of hypoxia-sensitive rainbow trout (*Oncorhynchus mykiss*) (Dunn and Hochachka, 1986),

which was reported to be incapable of mobilizing the reserve and, therefore, these hypoxia-sensitive fishes are not adapted to survive prolonged hypoxic condition.

In the results, we could not determine whether there is an energy partition between local (gill) and central (liver) storage. The glycogen in gill and liver degraded simultaneously at 6 h, even though there was a trend that the gill GP mRNA expression increased at 3 h. In two of the previous studies on tilapia (*Oreochromis mosssmbicus*), when acclimated in seawater or exposed to cadmium, the treated fish mobilized glycogen from liver to gill (Chang et al., 2007; Lin et al., 2011). Furthermore, in winter-acclimated crucian carp, the local glycogen (in brain, heart and gill) were transiently depleted under anoxia before the central reserve (in liver) were broken down to glucose for further systemic transport (Vornanen and Haverinen, 2011). It's also difficult to determine the partitions of liver glycogen, since the liver glycogen has to transfer to other organs (Chang et al., 2007). For example, *H. temminckii* increased its behavior of swimming into interface of air and water and gulping air during hypoxic exposure; therefore, the glycogen in muscle was consumed during swimming. *H. temminckii* swims vigorous to water surface and back to the bottom under hypoxia (personal observation). Previous study has shown that the muscle glycogen and liver glycogen both fell with increasing in intensity of swimming speed (Johnston and Goldspink, 1973ab; Coban and Sen, 2011), and the decreasing in liver glycogen implies the mobilization of glycogen to the swimming musculature (Johnston and Goldspink, 1973a). Accordingly, liver glycogen was degraded into glucose and mobilized to the organs which need energy such as gill and muscle in the *H. temminckii*. Since both ionoregulation and swimming are energy consuming activities and there is no glycogen provided additionally (fasted during experimental periods), the body glycogen will be outspent. Though the fish will utilize other energy sources such as protein, lipid and ketones under long-term stressful condition, they still have to reserve the energy-cost responses for surviving (Dave et al., 1975). Consequently, the NKA expression

may be inhibited after long-term hypoxia while *H. temminckii* exploited the energy resources. Furthermore, Kramer (1987) documented that a rise in breathing frequency which reduces the growth rate, since breathes is an energy-cost behavior and it decrease the energy available for growth. Several studies found that hypoxic condition impairs growth rate (Pichavant et al., 2000; Tran-Duy et al., 2008) and it is evident that the decrease in growth is due to the reduced food intake (Davis, 1975; Brett; 1979). However, the recently study revealed that muscle glycogen may be energy supplier because of a finding GP6ase in white myotomal muscle of crucian carp. It is, therefore, suggested that muscle tissues could contribute glucose converted from glycogen into blood (Vornanen et al., 2011). This finding could explain why hypoxia/anoxia-tolerant fish survive longer under hypoxia.

Antioxidant Defenses in Gill and Liver

The antioxidant enzymes that constitute the network of antioxidant defenses are expected to increase under hypoxia to detoxify the generation of ROS. Wilhelm Filho et al. (1993) found the activity of SOD was linearly correlated with CAT in six of the marine fishes. In our experiment, however, neither gills nor liver had such patterns. SOD activity increased in gill during reoxygenation at 1 h and catalyzed the dismutation of O_2^- into H_2O and H_2O_2, which is detoxified by CAT and GPx (Hermes-Lima et al, 2001). CAT activity in the gills was not significantly different but there was a trend for the decreased activity when exposed to hypoxia and reoxygenation. Such a decrease was concomitant with an increase in GPx in gill at 3 h in hypoxia and 72 h in recovery. A similar pattern was found when exposed rotan *Perccottus glenii* in 1 h recovery to normoxia (Lushchak and Bagnyukova, 2007). Woo et al. (2006) discussed about the expression of activity between CAT and GPx and the reasons of contrasting expression was unclear. It is proposed that the distribution was different between CAT and GPx. The CAT is mainly located in the peroxisomes, whereas GPx in the cytosol and mitochondrial. Therefore, the CAT in

peroxisome decomposes H_2O_2 generated from the breakdown of long chain fatty acid, while GPx decomposes H_2O_2 from mitochondria, endoplasmic reticulum or catalyzing by SOD (Woo, 2006). In addition, the pattern of SOD was reciprocally related to that of CAT but was directly proportional to GPx in the liver, suggesting the aerobic work on cellular respiration. Also, when exposed to hypoxia, spot *Leiostomus xanthus* had no detectable CAT activity in gill (Cooper et al., 2002). The gill may be releasing H_2O_2 by passive exchange, because of the high solubility and diffusion capacity in H_2O_2 (Wilhelm Filho et al., 1993; Marcon and Wilhelm Filho, 1999). The GSR activity was not significantly different but with a trend of increase in hypoxia and even higher in reoxygenation in gill. Moreover, GSR activity increased during hypoxia in liver. GSR is mainly responsible for recycling of GSSG into GSH which is the cosubstrate of GPx. Consequently, it is expected that GSR activity increased before an increase in GPx activity accordingly (Ahmad, 1995; Fridovich, 1998; Hermes-Lima and Zenteno-Savín, 2002), and this pattern was confirmed in the gills of *H. temminckii*. The GST activity increased in hypoxia at 3 h and 12 h reoxygenation in gill. GST is involved in the detoxification of xenobiotics and products of lipid peroxidation by catalyzing the conjugation of GSH (Singhal et al., 1992; Hermes-Lima and Storey, 1998) and therefore, increase in GST activity could reduce the contents of end products of lipid peroxidation in gill during hypoxia/reoxygenation (Lushchak et al., 2005).

Liver is the organ subjected to oxidative stress mostly during the transition from hypoxia to recovery condition. Elevated antioxidant enzymes in liver during exposure to hypoxia/anoxia and reoxygenation were documented in many species (Vig and Nemcsok, 1989; Hermes-Lima and Storey, 1993; Hermes-Lima and Storey, 1996; Lushchak et al., 2001; Cooper et a;., 2002; Lushchak et al., 2005; Luscchak and Bagnyukova, 2007), indicating the activation of protective mechanisms necessary to scavenge the ROS. Although there is no statistical difference in CAT and GPx activity in liver in the present

study, the CAT and GPx activity declined in some periods of hypoxia. Similarly, the results were found in the GPx activity in the liver of hypoxia-tolerant species common carp (*Cyprinus carpio*) (Lushchak et al., 2005). The reduction of antioxidant enzymes revealed the metabolic suppression in response to hypoxia and modified both protein synthesis and xenobiotic process (Lushchak et al., 2005). In addition to the antioxidant enzymes, thiobarbituric acid reactive substances (TBARS), lipid peroxides (LOOH) and protein carbonyl (CP), which are biomarkers of oxidative stress should be noted (Gutteridge, 1995; Dalle-Donne et al., 2003). In some studies, it was documented that the levels of TBARS, LOOH and CP are related to the arising of antioxidant enzymes. For example, when rotan *Perccottus glenii* was exposed to hypoxia and reoxygenation, the LOOH and CP levels increased during hypoxia and remained higher during recovery in liver and tissue, suggesting an elevation of the hypoxia-induced oxidative stress in these tissues (Lushchak and Bagnyukova, 2007). Compared to the previous study, the expressions of antioxidants in the present study are largely different in anoxia/hypoxia or reoxygenation. Trübenbach et al. (2012) found that some antioxidants enzyme only increase during reoxygenation and concluded that the antioxidants systems are highly species- or tissues-dependent.

Hermes-Lima proposed the hypothesis that increase in antioxidant defenses during anoxia/hypoxia to recovery (e.g. garter snakes *Thamnophis sirtalis* (Hermes-Lima and Storey, 1993); leopard frog *Rana pipiens* (Hermes-Lima and Storey, 1996); goldfish *Carassius auratus* (Lushchak et al., 2001)) estivation to arousal (e.g. land snails *Otala lactea* and *Helix aspersa maxima* (Hermes-Lima and Storey, 1995a, b)) and freeze to thaw (e.g. garter snake *Thamnophis sirtalis* (Hermes-Lima and Storey, 1993); wood frog *Rana sylvatica* (Joanisse and Storey, 1996)) is a key biochemical mechanism for preparative adaptation to oxidative stress (Hermes-Lima, 1998). However, evidence in skeletal muscle reflects that hypoxia induces ROS formation (Clanton, 2007). The further investigation of hypoxia-induced ROS in fish may be considered. In addition to induction of antioxidant

defenses by single factor, it is noteworthy to examine the combined-factor effects. Acclimation of pacu *Piaractus mesopotamicus* in hypoxia plus copper was found to have a combined effect to induce an ROS production followed by a rise in SOD activity in liver, which was not found when the factor was examined separately (Sampaio et al., 2008). Thus the combination of hypoxia and other toxicants may have potent impact than the effects of individual factor.

Appendix 2 is to summarize a series of responses of antioxidant enzyme under hypoxia and recovery phase. When O_2^- is produced from electron transport chain in mitochondria, SOD elevated to dismutase O_2^- into H_2O_2 (gill: recovery; liver: hypoxia). Then the H_2O_2 is decomposed by CAT (liver: recovery) in peroxisome and GPx (gill: hypoxia) in cytosol and mitochondria respectively. The GPx needs the GSH as the cosubstrate in the process of detoxification; therefore, the GSR is increased to convert the GSSG into GSH (liver: hypoxia). Finally, the GST is catalysis by conjugation with GSH to xenobiotics and the less toxic and more hydrophilic products will be metabolized and excreted (gill and liver: recovery).

Conclusions

To adapt to hypoxia, kissing gouramis *Helostoma temminckii* first adjust it behavior which enhances the frequency of air-breathing and modify the physiological condition accordingly. From the present study, it is suggested that the antioxidant enzymes increase immediately to prepare for the oxidative stress. The SOD activity rose during reoxygenation, and GPx and GST increased during hypoxia and reoxygenation in gill. In liver, the activity of SOD and GSR elevated during hypoxic exposure. Furthermore, the activity of CAT, GPx and GST in liver has the trend of increasing during recovery phrase. It implies that the antioxidant enzymes were elevated to prevent from the damage of reactive oxygen species. Within a short period of time, the fish degrade the glycogen to maintain the demand of physiological regulation. In the present study, the mRNA expression of GP did not decline accordingly, but the degrading of glycogen contents reflect transportation of glycogen from liver to the organs in need of fuels. Finally, by avoiding of depletion of energy resource rapidly, the organism has to inhibit the ionoregulation function. However, the expression profile of NKA in gill and kidney did not decline during hypoxia. There are two possible explanations; one is that protein synthesis was not suppressed in gill, and the other is that the glycogenolysis was performed in GR cells and transferred glucose to MR cells in order to preserve the osmoregulation in gills. The energy partition between gill and liver were not distinct; the mRNA and protein expression of GP in gill, however, rose at 3 h which was faster than the decreasing in glycogen content in liver.

Perspectives

In the lack of oxygen, some genes are induced to cope with the stressful condition. In rat brains, hypoxia induced the expression of glycogen phosphorylase (Gilany et al., 2011). In zebrafish, it is reported that the hypoxia-induced genes include, at least, lactate dehydrogenase (LDH) and hypoxia inducible factor (HIF) (Ton et al., 2003). HIF is a key modulator of the transcriptional response to hypoxic stress (Majmundar et al., 2010).and involves in up-regulation of the genes related to glycolysis (Seagroves et al., 2001), angiogenesis, cell proliferation and differentiation (Review by Benizri et al., 2008). The study on transcriptional regulation of Hela cells also showed the MCT4 is up-regulated through HIF-1α in plasma membrane (Ullah et al., 2006). Furthermore, HIF was stabilized by ROS during hypoxia (Chandel et al., 2000). According to the above contents, I modify the model from Taylor (2008) (Appendix 4). It is apparent that HIF is the major factor to connect each pathway. Although the effects of HIF on these pathways (induction of glycolysis, MCT4; stabilization by ROS) were not investigated in the present study, these pathways are awaited for further study to construct the detailed molecular system in air-breathing fishes exposing to hypoxia.

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Tables

Table 1 Water chemical properties and body length in the experimental group. Data are presented as mean ± S.E.M.

Chemical property/group	Normoxia	Hypoxia	Recovery
Dissolved Oxygen (mg/L)	8.40±0.10 (60)	0.81±0.02 (60)	8.61±0.28 (8)
pH value	7.55±0.05 (60)	7.61±0.03 (60)	7.64±0.10 (8)
Temperature (°C)	26.75±0.05 (60)	27.41±0.09 (60)	27.04±0.13 (8)
Body length (cm)	3.20±0.04 (172)	3.28±0.05 (165)	3.06±0.07 (16)

Table 2 Specific primer sets for quantitative real-time PCR

Gene name	Amplicon size bp	Sequence
NKA	217	Forward 5’-AGTGGCAGAAACCAAGTACTCTTT-3’ Reverse 5’-AATAAATACCAGCTCTCGATCCAC-3’
GP	205	Forward 5’-TACATGCTGCCAGTTCACTTCTAC-3’ Reverse 5’-GGATGTAATCTCCAACATTGAAGTC-3’
β-actin	210	Forward 5’-CCTCCGATCCAGACAGAGTATTA-3’ Reverse 5’-TGTCCATGAGACAACATACAACAG-3’

Figures

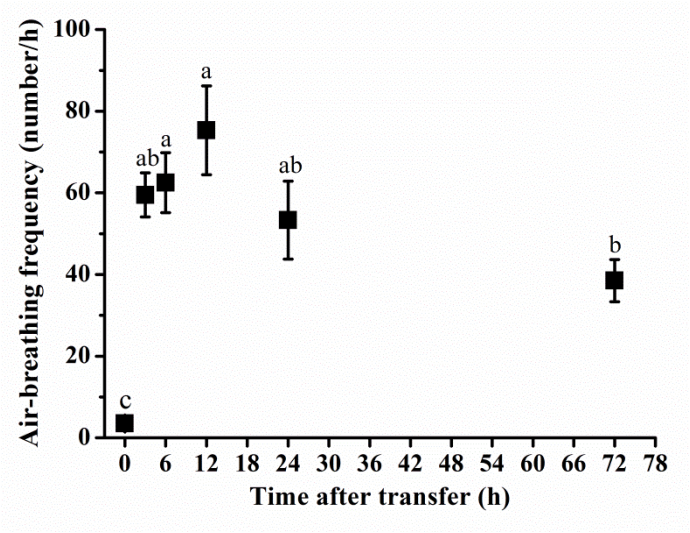


Figure 1 The air-breathing frequency of *Helostoma temminckii* exposed to normoxia and hypoxia. The air-breathing frequency under hypoxic condition was significantly higher at 3, 6, 12, 24, 72 h than that under normoxic condition. Data are presented as mean ± S.E.M. (n=6). The different letters indicate a significant difference among times (one-way ANOVA, Duncan's post hoc test, $p<0.05$).

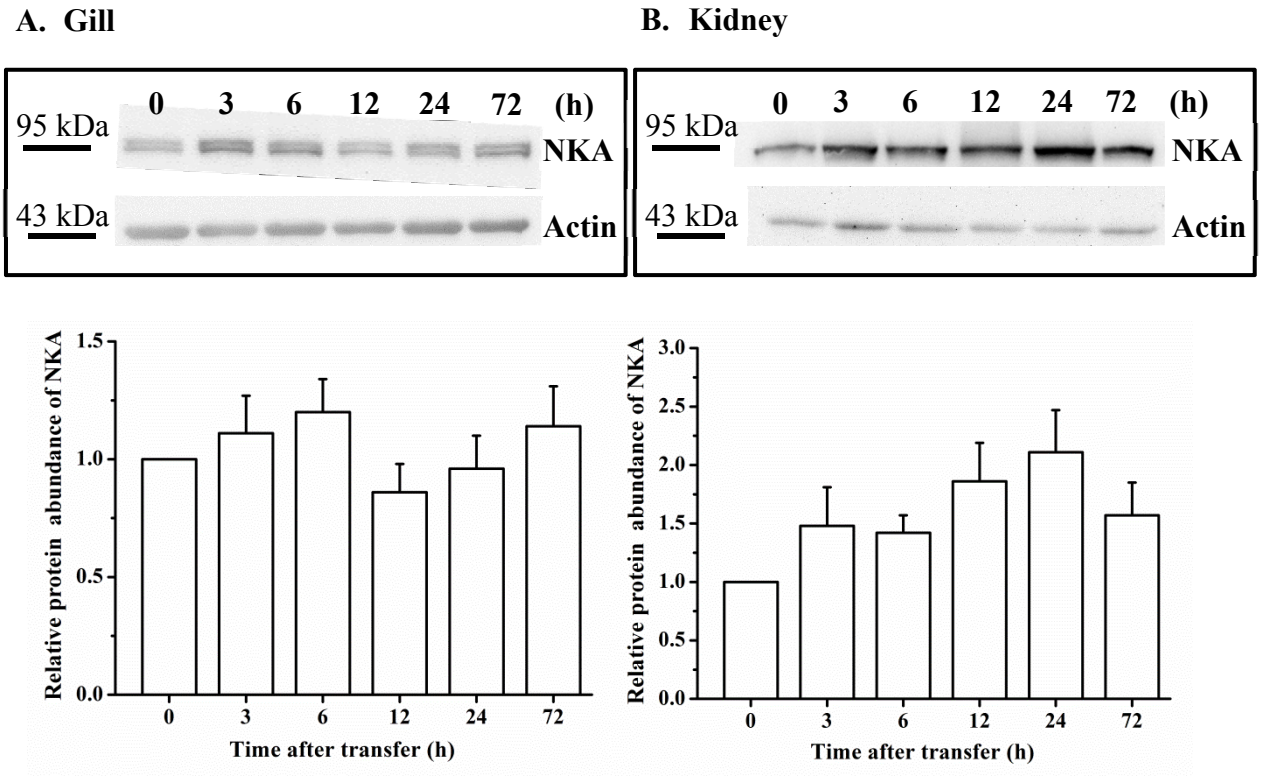


Figure 2 The time course change of relative protein abundance of NKA in the gill (A) and kidney (B) of *Helostoma temminckii* exposed to hypoxia. Two NKA immunoreactive bands were detected by immunoblots in the gill, while there was only one NKA immunoreactive band in the kidney. Data are presented as mean ± S.E.M. (n=8). One-way ANOVA was conducted among different treatments (p<0.05).

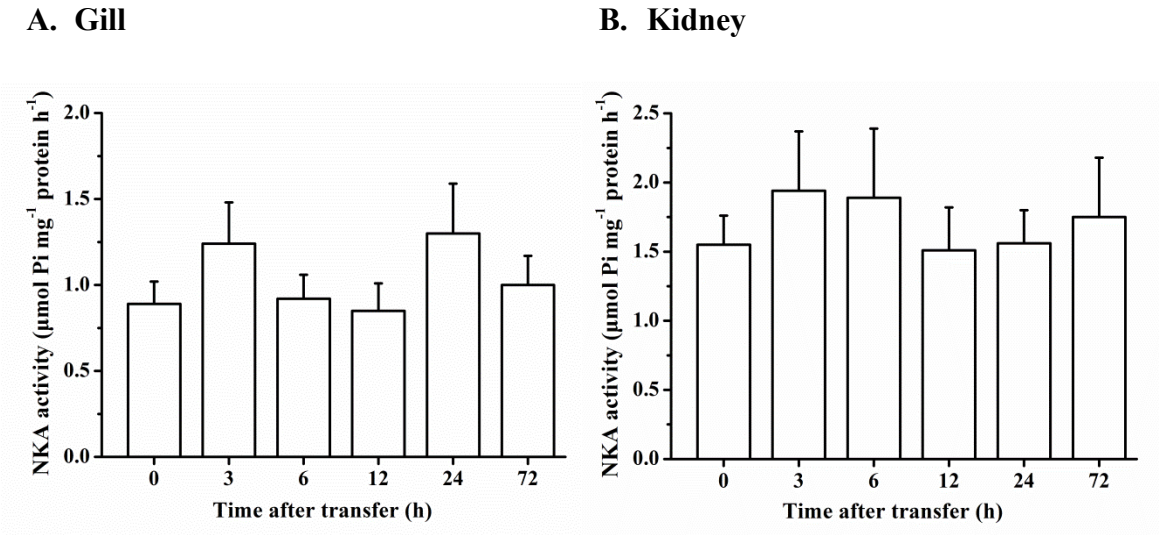


Figure 3 The time course change of NKA activity in the gill (A) and kidney (B) of *Helostoma temminckii* exposed to hypoxia. Data are presented as mean ± S.E.M. (n=8). One-way ANOVA was conducted among different treatments ($p<0.05$).

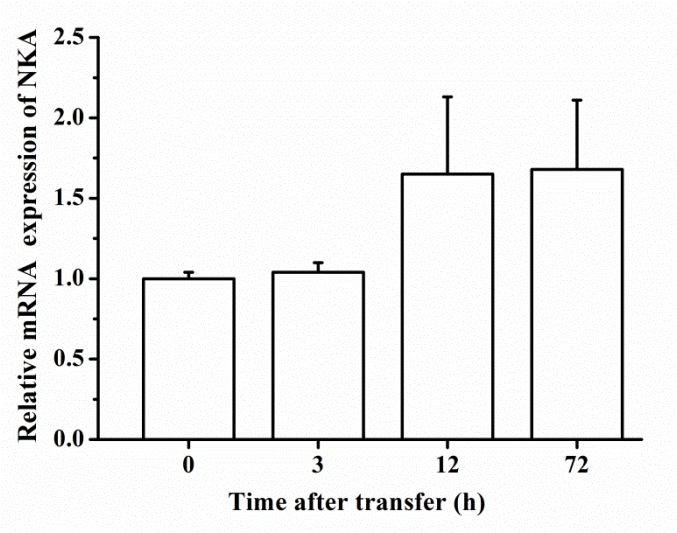


Figure 4 The time course change of relative mRNA expression of NKA in the gill of *Helostoma temminckii* exposed to hypoxia. Data are presented as mean ± S.E.M. (n=8). One-way ANOVA was conducted among different treatments ($p<0.05$).

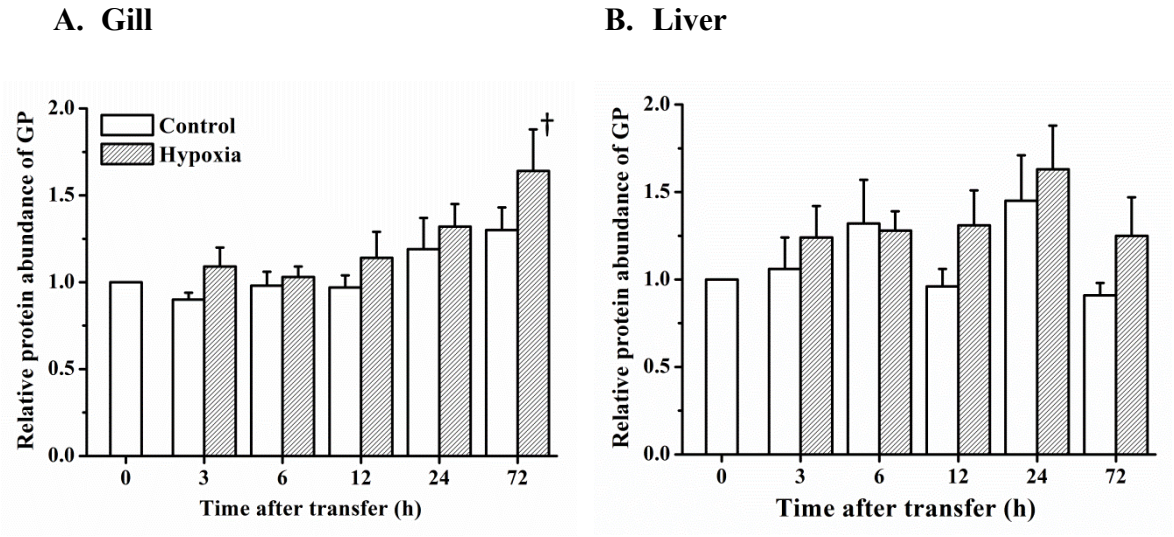


Figure 5 The time course change of relative protein abundance of GP in the gill (A) and liver (B) of H*elostoma temminckii* exposed to hypoxia. Data are presented as mean ± S.E.M. (n=8). One-way ANOVA was conducted among different treatments (p<0.05). †Indicates a significant difference compared with control (0 h) (one-way ANOVA, Dunnett's post hoc test, p<0.05).

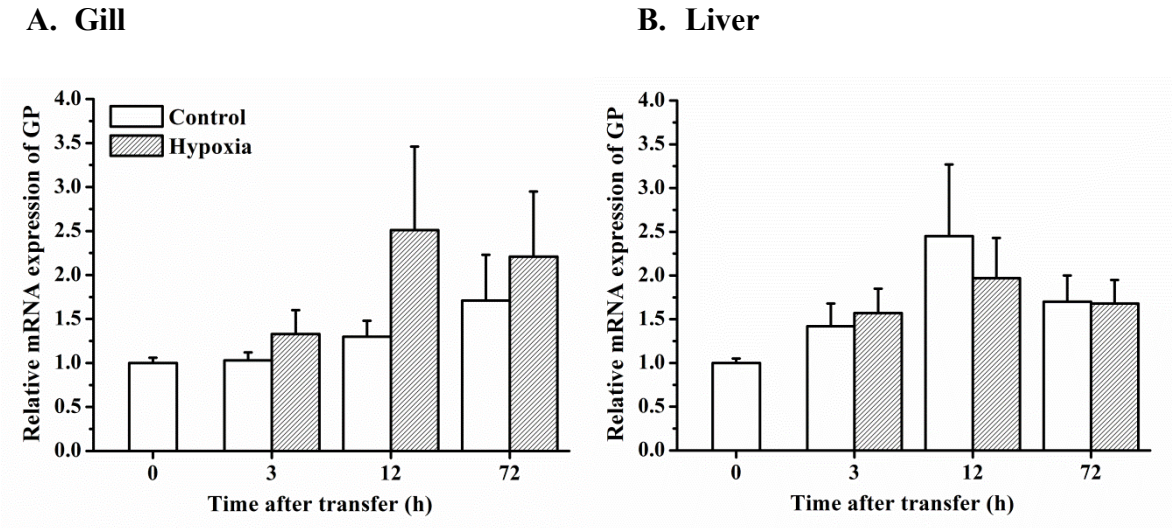


Figure 6 The time course change of mRNA relative expression of GP in the gill (A) and liver (B) of *Helostoma temminckii* exposed to hypoxia. Data are presented as mean ± S.E.M. (n=8). One-way ANOVA was conducted among different treatments (p<0.05).

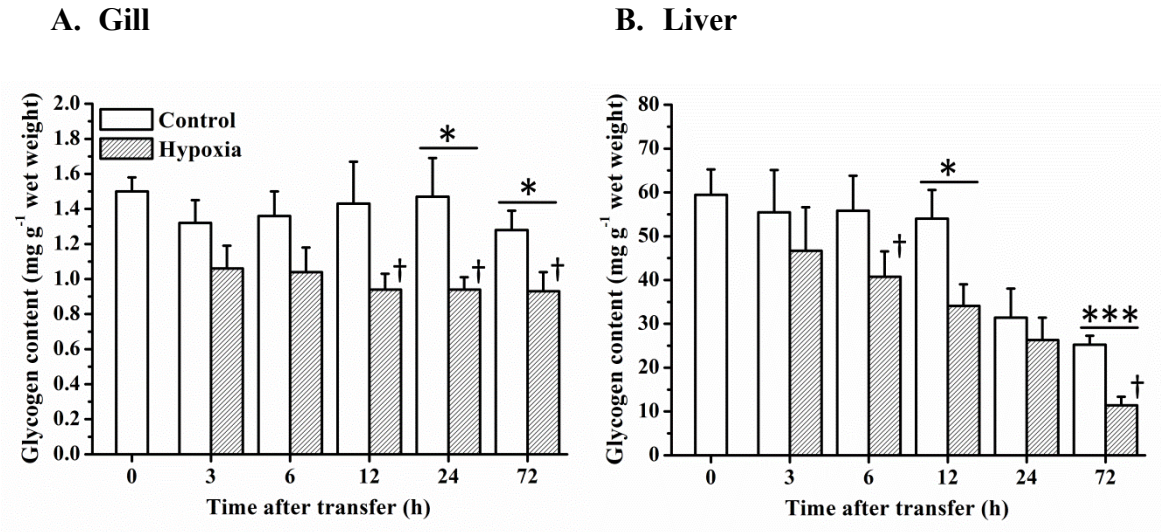


Figure 7 The time course change of glycogen content in the gill (A) and liver (B) of *Helostoma temminckii* exposed to hypoxia. Data are presented as mean ± S.E.M. (n=10). *Indicates a significant difference from the respective control at the same exposure time (Student's t-test, *: $p<0.05$, ***: $p<0.001$). †Indicates a significant difference compared with control (0 h) (one-way ANOVA, Dunnett's post hoc test, $p<0.05$).

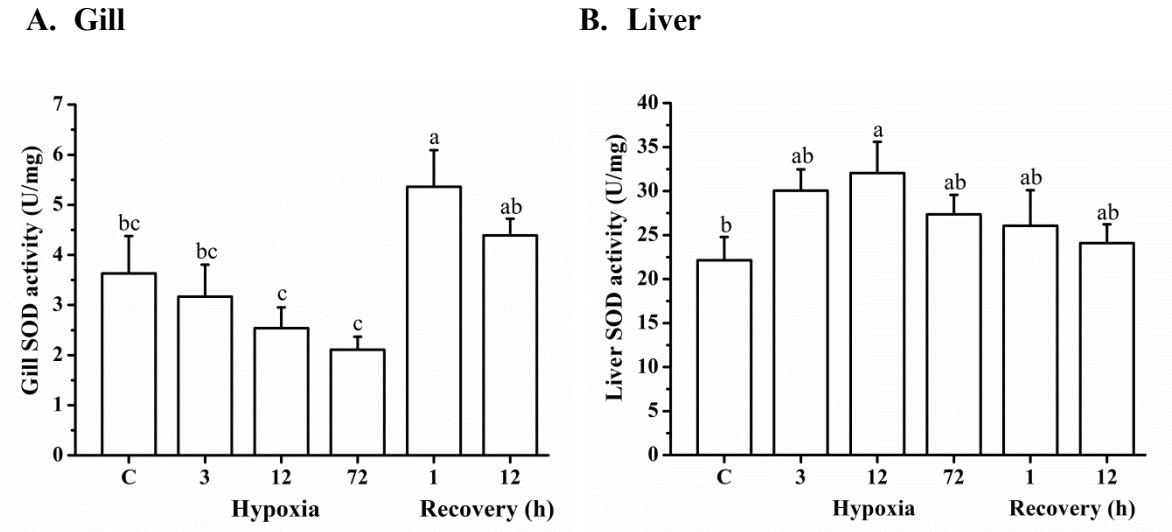


Figure 8 SOD activity under control (0 h), hypoxic condition at 3, 12 and 72 h and recovery to normoxia at 1 and 12 h in the gill (A) and liver (B) of *Helostoma temminckii*. Data are presented as mean ± S.E.M. (n=8). One-way ANOVA was conducted among different treatments ($p<0.05$). Different letters indicate a significant difference among sampling times of the treatment groups (one-way ANOVA, Duncan's post hoc test, $p<0.05$).

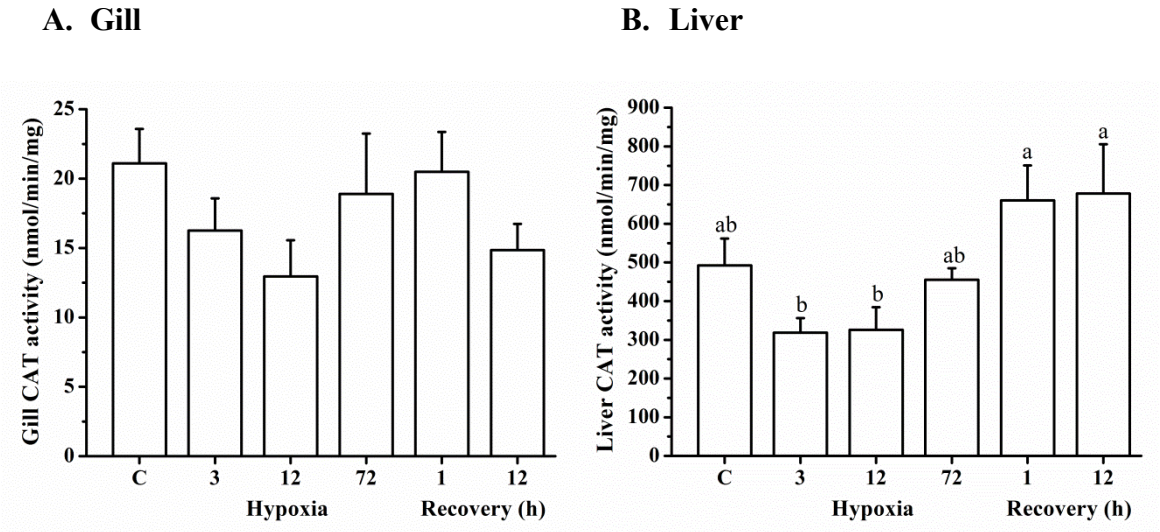


Figure 9 CAT activity under control (0 h), hypoxic condition at 3, 12 and 72 h and recovery to normoxia at 1 and 12 h in the gill (A) and liver (B) of *Helostoma temminckii*. Data are presented as mean ± S.E.M. (n=8). One-way ANOVA was conducted among different treatments (p<0.05). Different letters indicate a significant difference among sampling times of the treatment groups (one-way ANOVA, Duncan's post hoc test, p<0.05).

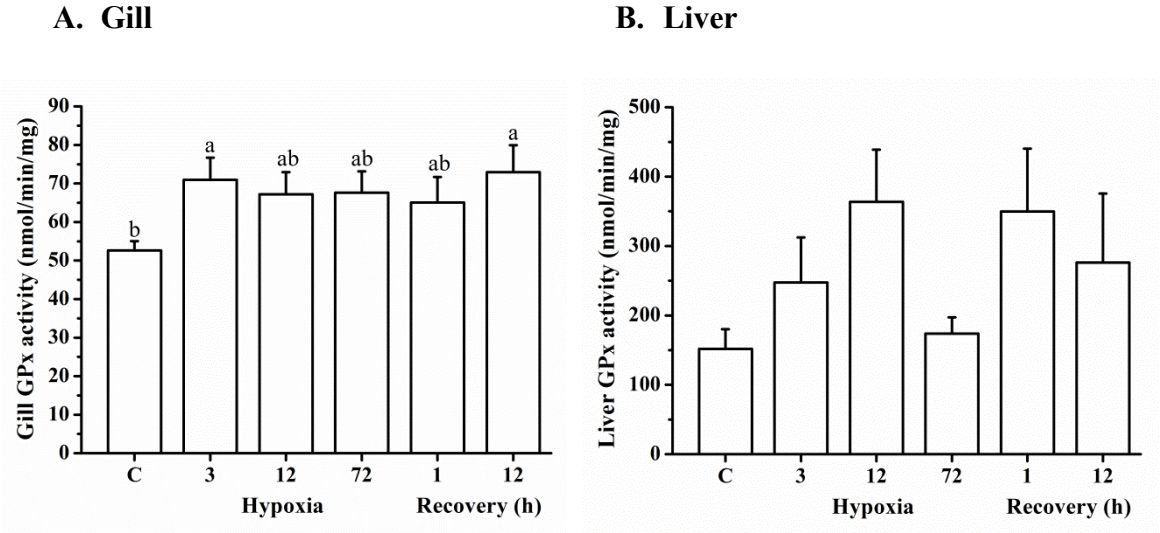


Figure 10 GPx activity under control (0 h), hypoxic condition at 3, 12 and 72 h and recovery to normoxia at 1 and 12 h in the gill (A) and liver (B) of *Helostoma temminckii*. Data are presented as mean ± S.E.M. (n=8). One-way ANOVA was conducted among different treatments ($p<0.05$). Different letters indicate a significant difference among sampling times of the treatment groups (one-way ANOVA, Duncan's post hoc test, $p<0.05$).

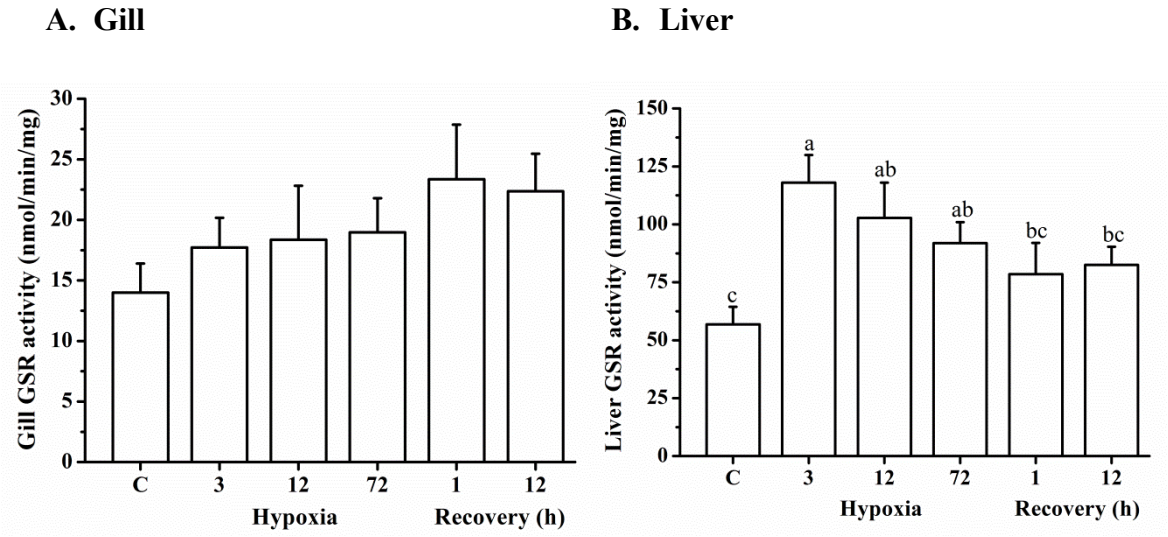


Figure 11 GSR activity under control (0 h), hypoxic condition at 3, 12 and 72 h and recovery to normoxia at 1 and 12 h in the gill (A) and liver (B) of *Helostoma temminckii*. Data are presented as mean ± S.E.M. (n=8). One-way ANOVA was conducted among different treatments ($p<0.05$). Different letters indicate a significant difference among sampling times of the treatment groups (one-way ANOVA, Duncan's post hoc test, $p<0.05$).

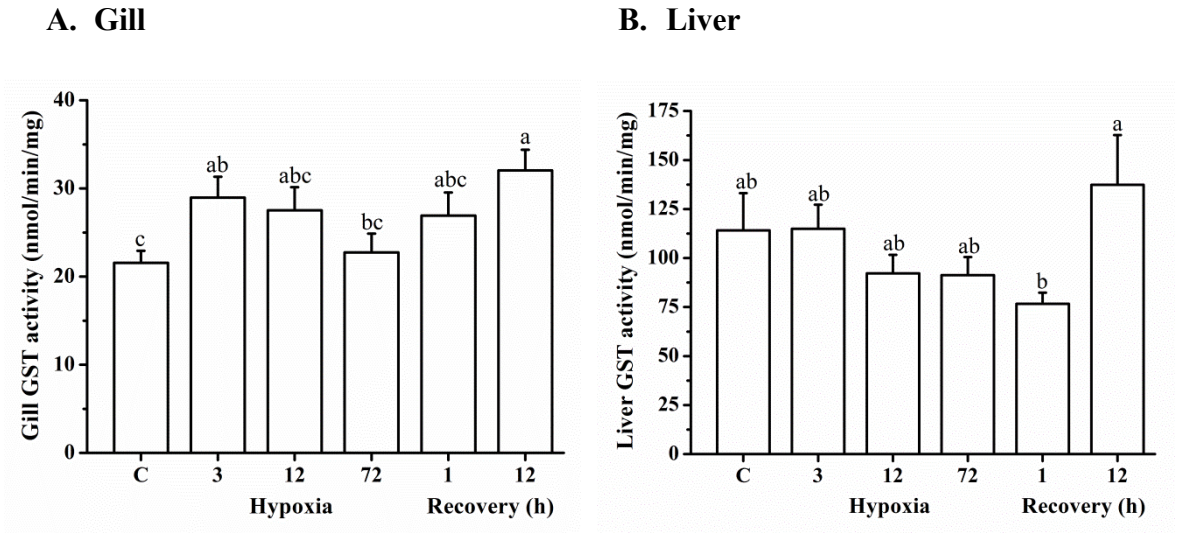
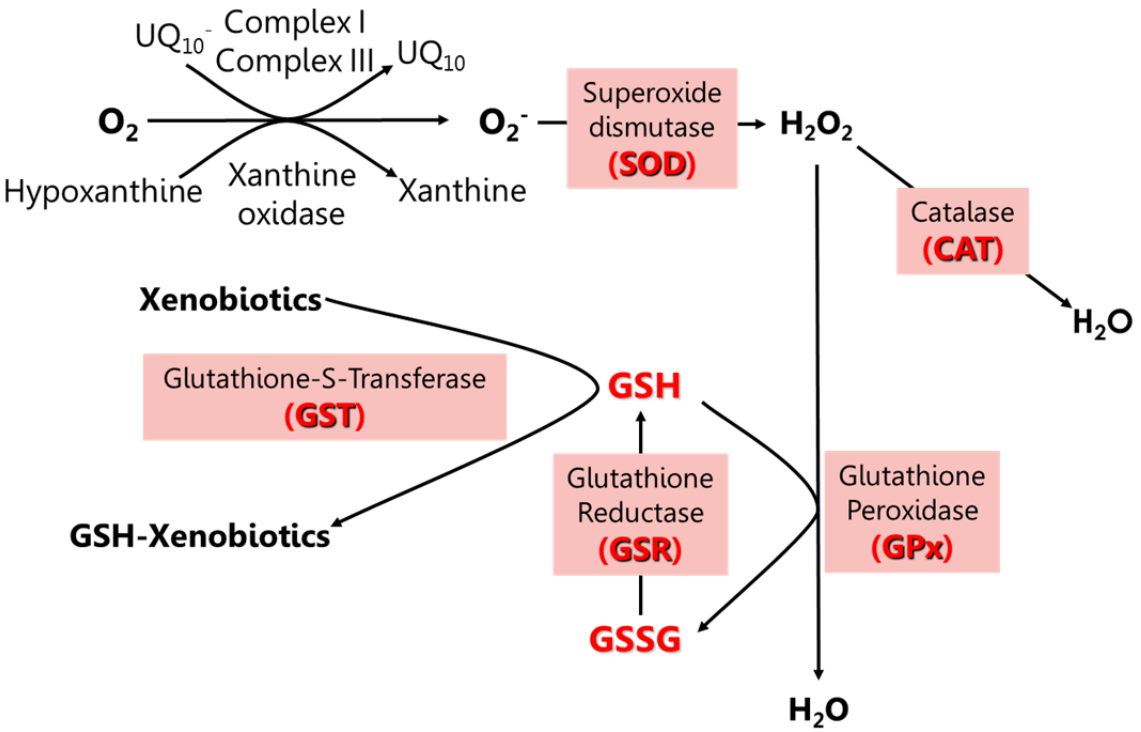


Figure 12 GST activity under control (0 h), hypoxic condition at 3, 12 and 72 h and recovery to normoxia at 1 and 12 h in the gill (A) and liver (B) of *Helostoma temminckii*. Data are presented as mean ± S.E.M. (n=8). One-way ANOVA was conducted among different treatments ($p<0.05$). Different letters indicate a significant difference among sampling times of the treatment groups (one-way ANOVA, Duncan's post hoc test, $p<0.05$).

Appendices



Appendix 1 Enzymatic antioxidant defense system of animal cell (modified from Raha and Robinson, 2000).

Appendix 2 The reactions of antioxidant enzymes.

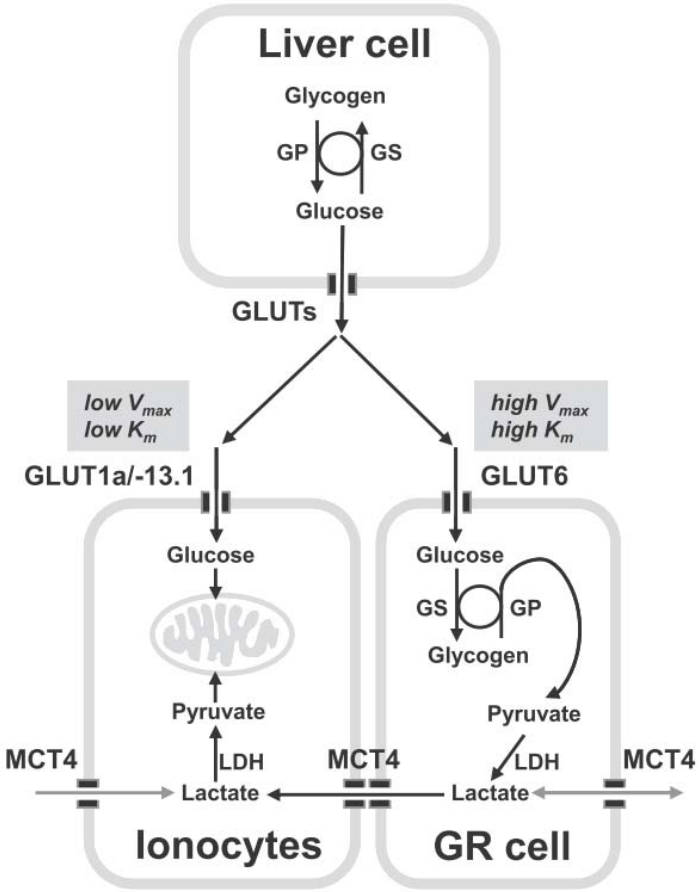
(1) $2O_2^- + 2H^+ \xrightarrow{\textbf{SOD}} H_2O_2 + O_2$

(2) $2H_2O_2 \xrightarrow{\textbf{CAT}} O_2 + 2H_2O$

(3) $ROOH + 2GSH \xrightarrow{\textbf{GPX}} ROH + GSSG + H_2O$

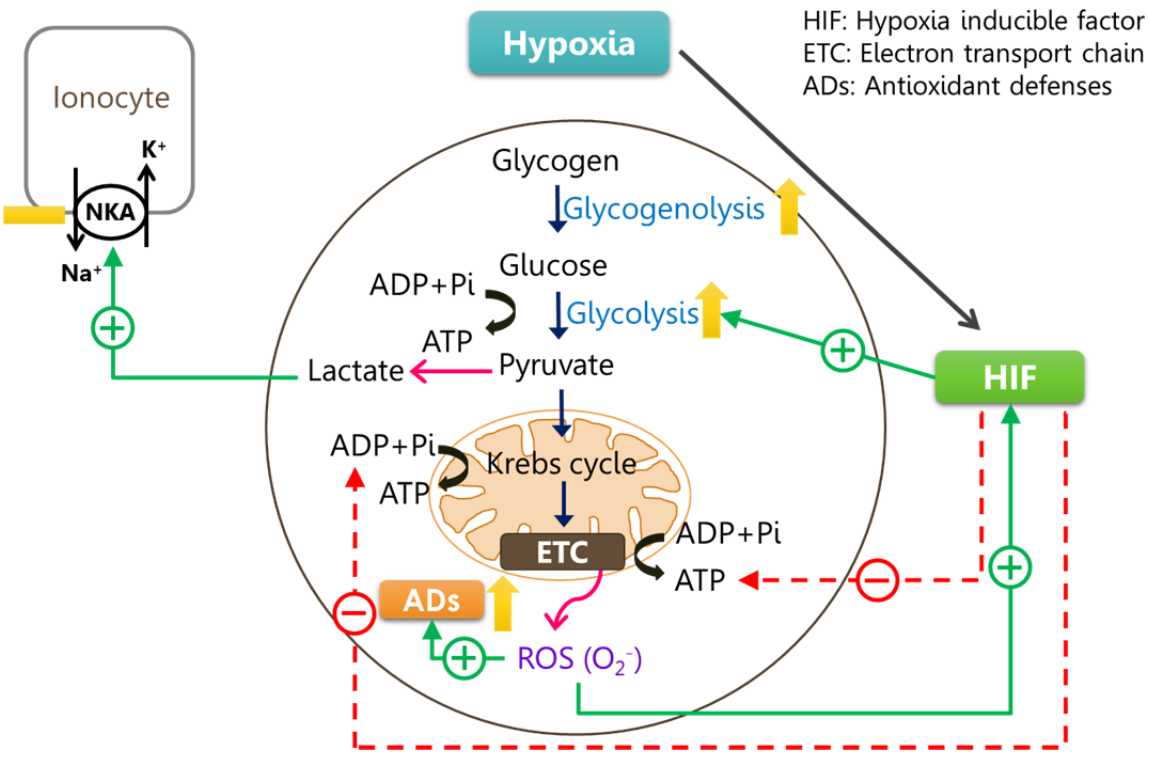
(4) $GSSG + NADPH + H^+ \xrightarrow{\textbf{GSR}} 2GSH + NADP^+$

(5) $GSH + CDNB \xrightarrow{\textbf{GST}} Dnp\text{-}SG$



Appendix 3 Model of the carbohydrate energy supply of ionocytes (Hwang et al., 2011).

GLUT: glucose transporter
GP: glycogen phosphorylase
GS: glycogen synthase
LDH: lactate dehydrogenase
MCT: monocarboxylate transporter
Dark gray line: unidentified transport pathway



Appendix 4 Proposed model for regulation of metabolism by HIF (modified from Taylor, 2008). Under hypoxia, HIF pathway promotes glycolysis pathway and the glycogenolysis increases. Glucose degrades to pyruvate and pyruvate converts into lactate. Lactate is transported to ionocyte and provides ATP-producing fuels. ROS is produced in mitochondria and induces antioxidant defenses. Moreover, the generation of ROS induces increase HIF expression.

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