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Effects of environmental hypoxia on cardiac energy metabolism and performance in tilapia

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Speers-Roesch B, Sandblom E, Lau GY, Farrell AP, Richards JG. Effects of environmental hypoxia on cardiac energy metabolism and performance in tilapia. *Am J Physiol Regul Integr Comp Physiol* 298: R104–R119, 2010. First published October 28, 2009; doi:10.1152/ajpregu.00418.2009.—The ability of an animal to depress ATP turnover while maintaining metabolic energy balance is important for survival during hypoxia. In the present study, we investigated the responses of cardiac energy metabolism and performance in the hypoxia-tolerant tilapia (*Oreochromis* hybrid sp.) during exposure to environmental hypoxia. Exposure to graded hypoxia (≥92% to 2.5% air saturation over 3.6 ± 0.2 h) followed by exposure to 5% air saturation for 8 h caused a depression of whole animal oxygen consumption rate that was accompanied by parallel decreases in heart rate, cardiac output, and cardiac power output (CPO, analogous to ATP demand of the heart). These cardiac parameters remained depressed by 50–60% compared with normoxic values throughout the 8-h exposure. During a 24-h exposure to 5% air saturation, cardiac ATP concentration was unchanged compared with normoxia and anaerobic glycolysis contributed to ATP supply as evidenced by considerable accumulation of lactate in the heart and plasma. Reductions in the provision of aerobic substrates were apparent from a large and rapid (in <1 h) decrease in plasma nonesterified fatty acids concentration and a modest decrease in activity of pyruvate dehydrogenase. Depression of cardiac ATP demand via bradycardia and an associated decrease in CPO appears to be an integral component of hypoxia-induced metabolic rate depression in tilapia and likely contributes to hypoxic survival.

fish; cardiovascular function; adenosine 5′-triphosphate; lipid; pyruvate dehydrogenase

DURING PERIODS OF LOW OXYGEN, hypoxia-tolerant animals undergo a profound, rapid, and reversible metabolic rate depression as shown by large decreases in oxygen consumption rate ($\dot{M}O_2$) and heat production (44, 54). This metabolic rate depression reflects a downregulation of cellular ATP turnover to a level that can be sustained by oxygen-independent ATP production. The ability to balance ATP demand with supply and thus maintain stable cellular ATP concentration ([ATP]) is a key response ensuring hypoxic survival in tolerant animals, including many species of fishes that regularly encounter environmental hypoxia (7).

A major component of the hypoxia-induced depression of ATP turnover is a reduction of cellular ATP demand, including the regulated arrest of ion pumping and anabolic pathways such as protein synthesis (30, 45, 49). Metabolic control analyses demonstrate, however, that ATP turnover in both active and metabolically depressed organisms can be controlled both by regulating ATP demand as well as by modulation of metabolic pathways involved in ATP supply such as mitochondrial substrate oxidation (6, 49). Our knowledge is incomplete as to how processes of ATP demand and ATP supply respond during hypoxia exposure in order to achieve depressed ATP turnover and stable cellular [ATP], including how these responses depend on the species and tissue investigated (49).

The heart of hypoxia-tolerant fishes represents a good system to study the metabolic balance of processes involved in ATP supply and demand during hypoxia exposure because it is a vital, highly aerobic tissue and its ATP demand can be readily quantified by measuring cardiac power output (CPO). CPO is the product of cardiac output (Q) and ventral aortic blood pressure (P_{VA}) and represents the mechanical output of the heart, which is the main source of cardiac ATP consumption (20). Reductions in CPO during exposure to environmental hypoxia have been observed in the hypoxia-tolerant common carp (*Cyprinus carpio*) and the anoxia-tolerant turtle *Trachemys scripta* (20). In these species, the depression of CPO is primarily mediated by cholinergic bradycardia and an associated decrease in Q and serves to reduce cardiac ATP demand to levels that can be sustained by maximal glycolytic ATP production alone (20). This strategy for hypoxic depression of cardiac ATP turnover contrasts with what is seen in the anoxia-tolerant crucian carp (*Carassius carassius*), in which routine CPO is kept at levels below the maximal glycolytic capacity even under normoxic conditions, obviating the need to reduce CPO during hypoxia (51). The degree to which these schemes generally apply to hypoxia-tolerant fishes is uncertain because CPO often is not measured and cardiovascular responses to hypoxia exposure vary among species studied (21). For example, hypoxia-induced cholinergic bradycardia is not found in all fishes, and compensatory increases in stroke volume (V_{SH}) and changes in P_{VA} are species dependent, resulting in variation in how Q and CPO in different fishes respond to hypoxia (19, 21). The degree to which changes in these parameters relate to whole animal hypoxia-induced metabolic rate depression is also unclear. Studies on certain teleosts show that the oxygen tension at the onset of hypoxic bradycardia corresponds well with the point where $\dot{M}O_2$ transitions from being independent of environmental oxygen tension to being dependent [quantified as critical oxygen tension (P_{crit})] and the depression of $\dot{M}O_2$ and heart rate (f_H) are closely linked (22, 40).

Our understanding of how ATP supply pathways in the heart of hypoxia-tolerant animals respond during periods of low oxygen is incomplete. It is known that glycolysis is essential

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for hypoxic function of the fish heart (21). To what extent aerobic ATP supply pathways, including glucose oxidation and fatty acid oxidation, are modulated during hypoxia in the heart of tolerant fishes is unknown, despite the potential importance of such responses in controlling depression of ATP turnover during hypoxia exposure (6, 49), as well as preventing the possible accumulation of harmful by-products of inefficient mitochondrial respiration such as reactive oxygen species (ROS) and fatty acid intermediates (12, 13). Richards et al. (44, 45) showed that the activity of pyruvate dehydrogenase (PDH), the key regulatory point of pyruvate entry into the TCA cycle and mitochondrial oxidation, decreased in white skeletal muscle of two hypoxia-tolerant teleosts, the oscar (*Astronotus ocellatus*) and the killifish (*Fundulus heteroclitus*), during hypoxia exposure. The decrease may be mediated by PDH kinase-2 (PDK-2; Ref. 44), one of four PDKs found in mammals and fishes that phosphorylate and inactivate PDH. Normally this occurs when cellular energy status is high (e.g., high [acetyl CoA]/[CoA]; Ref. 26), but hypoxia-inducible factor (HIF)-mediated increases of PDKs during hypoxia exposure have been shown to reduce oxygen consumption and ROS formation and contribute to hypoxia tolerance in mammalian cells and tissues (2, 48). Less is known about the responses of fatty acid oxidation in hypoxia-tolerant fishes exposed to hypoxia, but a norepinephrine-mediated decrease in circulating nonesterified fatty acids (NEFA) appears to be a common response to oxygen deprivation that may limit fatty acid oxidation in hypoxia-tolerant fishes (56, 57, 60). A regulated decrease in fatty acid oxidation could contribute to reduced ATP turnover and possibly avert detrimental effects of fatty acid oxidation in hypoxia. These effects, which are well characterized in mammalian ischemic heart models, include lipotoxicity and uncoupling of glycolysis from glucose oxidation that exacerbates metabolic acidosis and disturbs ion homeostasis (16). Interestingly, this latter event is mediated by AMP-activated protein kinase (AMPK), which is potentially important in decreasing $\dot{M}O_2$ and ATP demand in certain tissues in goldfish (*Carassius auratus*) and crucian carp during oxygen lack (28, 52).

In this study we investigated how exposure to environmental hypoxia affects cardiac ATP demand and metabolic fuel selection in the hypoxia-tolerant tilapia (*Oreochromis* hybrid sp.). Cardiac ATP demand and whole animal metabolic rate depression were quantified by monitoring cardiovascular parameters and $\dot{M}O_2$, respectively, during graded hypoxia (>92% to 2.5% air saturation over 3.6 ± 0.2 h) followed by exposure to 5% air saturation for 8 h. In a separate 24-h hypoxia exposure (5% air saturation), plasma and heart were sampled and components of certain ATP supply pathways were assessed. In heart, we measured AMPK activity, markers of oxygen-independent ATP production [lactate, creatine phosphate (CrP)], and energetic status (ATP, ADP_{free}, AMP_{free}). Control of carbohydrate oxidation was assessed via analysis of PDH activity and expression of PDK-2, and fatty acid oxidation was investigated by measuring concentrations of carnitine esters and mRNA levels of carnitine palmitoyltransferase-1 (CPT-1), the rate-limiting step in mitochondrial fatty acid oxidation, and peroxisome proliferator-activated receptor (PPAR)α, a key transcriptional regulator of genes related to fatty acid oxidation. Plasma was assayed for circulating levels of glucose and NEFA to indicate how substrate supply is altered during hypoxia exposure. Together, these experiments provide insight into how ATP supply and demand is modulated in the heart of tilapia during hypoxia exposure.

MATERIALS AND METHODS

Animals

Adult male tilapia (*Oreochromis niloticus* × *mossambicus* × *hornorum*; strain origin: Ace Developments, Bruneau, ID) were purchased from Redfish Ranch (Courtenay, BC, Canada). Fish were kept at the University of British Columbia in well-aerated 400-liter tanks supplied with recirculating freshwater (22°C). Fish were held for at least 2 mo before experimentation and fed daily to satiation with commercial trout pellets (FirstMate, Taplow Aquaculture, North Vancouver, BC, Canada). All experiments were conducted according to guidelines set out by the Canadian Council for Animal Care and protocols approved by the University of British Columbia Animal Care Committee.

Experiments

Three experiments were performed to characterize the responses of tilapia hearts to hypoxia exposure. First (*series I*), we assessed the hypoxia tolerance of tilapia by measuring $\dot{M}O_2$ and P_{crit} via closed respirometry as well as whole blood hemoglobin-O_2 binding affinity (P_{50}). Second (*series II*), we measured cardiovascular responses during graded hypoxia followed by exposure to severe hypoxia (5% air saturation) for 8 h to evaluate the effects of hypoxia on ATP demand of the heart. Simultaneous measurement of $\dot{M}O_2$ during graded hypoxia allowed us to calculate P_{crit} and investigate the relationship between depression of $\dot{M}O_2$ and cardiovascular status. Third (*series III*), during a 24-h hypoxia exposure (5% air saturation) we investigated pathways of ATP supply in the heart by measuring the products of oxygen-independent ATP production as well as key biochemical and molecular parameters contributing to the regulation of fatty acid and carbohydrate oxidation.

Series I. Assessment of Hypoxia Tolerance

Routine oxygen consumption rate ($\dot{M}O_2$) and P_{crit}, the inflection point at which $\dot{M}O_2$ ceases to be independent of water partial pressure of oxygen (PO_2), were determined at 22°C by closed respirometry according to the methods of Henriksson et al. (25). Briefly, individual fish (n = 7, 185 ± 2.9 g) were placed in a 10-liter swim tunnel respirometer (Loligo Systems, Tjele, Denmark) and allowed to acclimate under flow-through conditions for 12 h, at which point the respirometer was sealed and water PO_2 was continuously recorded with a fiber-optic oxygen sensor (Foxy system; Ocean Optics, Dunedin, FL) until 6% air saturation was reached. The swim tunnel propeller was run slowly throughout the trial to provide for adequate water mixing within the respirometer without swimming being necessary. All fish remained quiescent throughout the trials, each of which lasted ~6 h. $\dot{M}O_2$ and P_{crit} were calculated as described by Henriksson et al. (25), and the values of $\dot{M}O_2$ presented in Fig. 1 are means of individual $\dot{M}O_2$ calculated from 5-min intervals that occurred at specific PO_2 ± 2.5% air saturation between ~70% and 6% air saturation. A blank was run without a fish in the chamber to calculate background $\dot{M}O_2$, and this value was subtracted from fish measurements.

Tilapia whole blood hemoglobin-O_2 P_{50} was measured on freshly sampled blood from normoxic anaesthetized fish at physiological temperature (22°C) with a custom-made PWee50 according to the methods described by Henriksson et al. (25).

Series II. Cardiac ATP Demand

Surgical procedures. Fish (709.4 ± 25.2 g, n = 6) were netted from the holding tanks, anesthetized in water containing $NaHCO_3$-

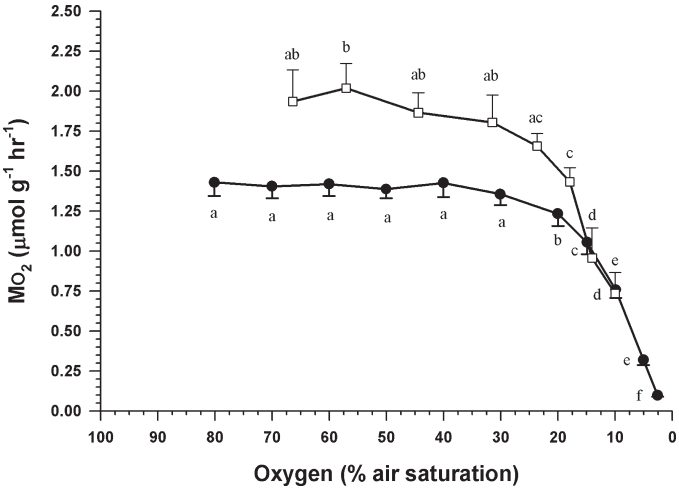

Fig. 1. Oxygen uptake ($\dot{M}O_2$) of tilapia during progressive decreases in water oxygen content from 66% air saturation to 10% air saturation measured during initial assessment of hypoxia tolerance (*series I*; □, $n = 7$), or from 80% air saturation to 2.5% air saturation measured simultaneously with cardiovascular variables (*series II*; ●, $n = 6$) (100% air saturation = 156 Torr = 20.8 kPa). Data are means ± SE. Values that do not share a letter are significantly different from other values within each series ($P < 0.05$) (letters for *series I* and *series II* are above and below data points/line, respectively).

buffered MS-222 (0.2 g/l and 0.2 g/l, respectively), and moved to a surgery table, where the gills were continuously irrigated via the mouth with chilled aerated water (13°C) containing $NaHCO_3$-buffered MS-222 (0.2 g/l and 0.15 g/l, respectively).

To permit measurement of P_{VA}, the left third afferent branchial artery was occlusively cannulated (4) with a PE-50 or PE-30 catheter filled with heparinized (100 IU/ml) glucose-free Cortland saline. The tip of the catheter was advanced toward the ventral aorta and secured in place by tying the catheter to the gill arch with 2-0 silk suture and then suturing the free end of the catheter to the skin. Correct placement of the catheter was verified by withdrawal of blood and by postmortem dissection of several fish. To measure ventral aortic blood flow (i.e., Q), the ventral aorta was exposed at the isthmus and a 2.5S Transonic transit-time blood flow probe (Transonic Systems, Ithaca, NY) was positioned around the vessel. The lead from the flow probe was tied to the skin with 2-0 silk. After surgery, fish were revived in freshwater at 22°C and placed in individual holding containers that were floating in a larger holding tank. Fish were allowed at least 24 h of recovery after instrumentation because this period of recovery was found to be sufficient to allow return of cardiovascular function to baseline levels (data not shown).

Experimental setup and protocol. The instrumented fish was moved to an acrylic glass respirometer (10 liters, Loligo Systems) that was submersed in a larger outer glass aquarium fed with the recirculating water system (22°C) that ensured normoxic (>90% air saturation) conditions. A submersible pump created a continuous flow of water from the external tank to the respirometer that was sufficient to ensure complete mixing inside the respirometer but negated the need for active swimming movements to maintain position. The aquarium was covered with polystyrene foam to prevent visual disturbance of the fish. The respirometer could be closed by recirculating water inside the respirometer with the submersible pump. The catheter and the flow probe lead from the fish were exteriorized through a hole in the respirometer fitted with a soft rubber stopper modified with a slit. The fish was allowed to habituate to the respirometer for at least 12 h before any experimental procedures.

Routine cardiovascular variables were continuously recorded in normoxia (≥92% air saturation) for several hours to ensure stable baseline conditions. The respirometer was then closed, and $\dot{M}O_2$ was measured from the rate of depletion of water P_{O_2} due to fish respiration. Once the nadir in water P_{O_2} was reached (~2.5% air saturation, which took 3.6 ± 0.2 h), P_{O_2} was increased to 5% air saturation within <5 min by manual adjustment of a siphon connected to the respirometer, which allowed a minimal flow of normoxic water to enter the respirometer to maintain P_{O_2}. The fish was then held for 8 h at this level of hypoxia, which was chosen on the basis of our measurements of P_{crit} and hemoglobin P_{50} (see RESULTS). Preliminary experiments showed that tilapia survived at least 24 h at 5% air saturation at 22°C. After the 8-h hypoxia exposure, normoxic water was reintroduced to the respirometer and measurements of cardiovascular variables were made for an additional 1.5 h of recovery in normoxic water. In one fish, blockage of the pressure cannula at ~7 h precluded further recordings of P_{VA}, so $n = 5$ for 8 h and recovery points. Fish were killed at the end of the trial, and the ventricle was excised, emptied of blood, blotted dry, and weighed.

Because of the use of closed respirometry, $\dot{M}O_2$ was not measured at the initial normoxic P_{O_2} (~92% air saturation) or the recovery period where flow-through conditions were used, nor was $\dot{M}O_2$ measured during the 8-h hypoxia exposure when water P_{O_2} was kept at 5% air saturation. The use of closed respirometry raises the question of whether changes in water parameters other than oxygen (e.g., pH, P_{CO_2}) could have had significant effects on measured parameters. This is unlikely for several reasons. Despite modest decreases in water pH and modest increases in water P_{CO_2} in closed respirometry trials of great sculpin (*Myoxocephalus polyacanthocephalus*), P_{crit} is not different from the same trials run under partial flow-through conditions where P_{CO_2} and pH were unchanged (J. G. Richards, unpublished observation). Additionally, in another hypoxia-tolerant cichlid the increases in water and blood P_{CO_2} are small during closed respirometry P_{crit} trials (47). A similar increase in environmental P_{CO_2} has been shown to cause only a very modest change in f_H and no change in Q in the sensitive rainbow trout (*Oncorhynchus mykiss*) (34). Similarly, studies on other hypoxia-tolerant fishes show only modest effects of high CO_2 on f_H (41). Thus the small increases in P_{CO_2} and pH expected in our study are unlikely to have contributed to the observed changes in cardiovascular parameters. During the 8-h exposure to 5% air saturation (*series II*) further changes in water properties are unlikely because the fish metabolic rate was greatly depressed, freshwater was bled into the respirometer, and measured parameters were stable.

Data acquisition and calculation of cardiorespiratory variables. The ventral aortic catheter was connected to a pressure transducer (model DPT-6100, pvb Medizintechnik, Kirchseeon, Germany) that was calibrated against a static water column, with the water surface in the experimental tank serving as zero-pressure reference. The signal from the transducer was amplified with a 4ChAmp amplifier (Somedic, Hörby, Sweden). Blood pressure recordings made in the respirometer were compensated for the small change in pressure (~0.5 kPa) that occurred in the respirometer depending on whether it was in an open or closed state. Cardiac output was recorded with a Transonic blood flowmeter (model T206, Transonic Systems). All flow probes used in the experiment were calibrated according to manufacturer guidelines at 22°C after the experiment to compensate for the effect of calibration temperature on flow readings. Water P_{O_2} in the respirometer was measured with an Oxyguard probe (Mark IV, Point Four Systems, Richmond, BC, Canada), modified to give a ±1-V output signal, that was placed in a custom-made Plexiglas chamber connected in line with the circulation pump. All signals were fed into a Power Lab unit (ADInstruments, Castle Hill, Australia) and subsequently analyzed with LabChart Pro software (v. 6.0, ADInstruments).

Cardiovascular parameters were analyzed in LabChart Pro at discrete oxygen tensions from ~92% air saturation to 2.5% air saturation by averaging the data across the time span bracketed by each tension ± 2.5% air saturation (i.e., the measurement of Q at 60% air saturation represents an average of Q recorded from 62.5% to 57.5% air saturation). For measurements at 5% and 2.5% air saturation, data

were averaged between ~7% and 3.5% and 3.5% and 2% air saturation, respectively. Whole animal $\dot{M}_{O_2}$ was calculated from the rate of decline in water P_{O_2} over the same discrete oxygen tensions where the cardiovascular parameters were collected (corrected for fish weight and respirometer volume), and P_{crit} was calculated as described above. Q was calculated directly from the flow trace in LabChart Pro. P_{VA} was calculated with the blood pressure analysis module in LabChart Pro. f_H was calculated from the pulsatile pressure or flow trace. V_{SH} was calculated as Q/f_H, and total peripheral resistance (R, i.e., the sum of systemic and branchial resistance) was calculated as P_{VA}/Q, with the assumption that central venous blood pressure is zero. CPO (mW/g wet ventricular mass) was calculated as the product of P_{VA} (kPa) and Q (ml/s) divided by the wet ventricular mass (g). Cardiovascular parameters were plotted against water P_{O_2} to identify the inflection points at which each parameter ceased to be independent of water P_{O_2} (i.e., P_{crit} of each cardiovascular parameter) as described above for calculation of P_{crit} of $\dot{M}_{O_2}$.

Series III. Cardiac ATP Supply

Gene identification, sequencing, and tissue distribution. Tilapia ($n = 3$) were sampled directly from normoxic holding aquaria and euthanized with benzocaine solution (100 g/l ethanol). Samples of heart, liver, red muscle, white muscle, kidney, adipose tissue, intestine, blood, and brain were dissected from the fish, immediately frozen in liquid nitrogen, and stored at −80°C. Total RNA was extracted from tilapia tissues with Tri reagent (Sigma-Aldrich, St. Louis, MO) and then quantified spectrophotometrically, and its integrity was verified via electrophoresis on a 1.5% agarose gel containing ethidium bromide.

First-strand cDNA was synthesized from 5 μg of total RNA isolated from the above tissues as described by Richards et al. (44). cDNA from heart was screened to identify CPT-1, PPARα, and PDK-2 gene isoforms expressed in tilapia heart. Tilapia partial CPT-1 sequences were obtained with degenerate primers (Table 1) determined from conserved regions of CPT-1α from horse (accession no. AB188099), sheep (NM001009414), human (L39211), rat (NM031559), mouse (BC054791), chicken (AY675193), and rainbow trout (AF327058). A partial PPARα sequence was obtained with degenerate primers (Table 1) determined from conserved regions of PPARα from *Salmo salar* (AM230809.1), *Sparus aurata* (AY590299.1), rat (NM013196.1), and chicken (NM001001464.1). Tilapia partial PDK-2 sequences were obtained with degenerate primers (Table 1) determined from conserved regions of PDK-2 from zebra fish (NM200996.1), mouse (NM133667.1), rat (NM030872.1), cow (BT025357.1), and *Xenopus laevis* (BC110980.1). Primers were designed with the assistance of GeneTool Lite software (www.biotool.com). Polymerase chain reactions (PCR) were carried out in a PTC-200 MJ Research thermocycler with *Taq* DNA polymerase (MBI Fermentas) and heart cDNA. Each PCR consisted of an initial 2 min at 94°C, followed by 40 cycles of 0.5 min at 94°C; 0.5 min at 54°C (CPT-1), 56°C (PPARα), or 47°C (PDK-2); and 1.5 min at 72°C. PCR products were electrophoresed on 1.5% agarose gels containing ethidium bromide to verify the amplification of product of the correct size.

Cloning of PCR products was carried out according to the methods of Richards et al. (44). Plasmids containing the PCR product were sequenced at the NAPS core facility at the University of British Columbia. Multiple clones of each cDNA fragment were sequenced in both directions at least twice, and a majority-rule consensus for the cDNA transcript was developed for each isoform. The BLAST algorithm was used to compare the cDNA sequences with published sequences in GenBank, and multiple alignments were produced with ClustalW. cDNA sequences have been deposited into GenBank (*CPT_{Til}−1 iso1*, Bankit 1241880; *CPT_{Til}−1 iso2*, Bankit 1241881; *PDK_{Til}−2*, Bankit 1241877; *$PPAR_{Til}$α*, Bankit 1241879).

The relative tissue distribution of mRNA expression of the genes was ascertained by quantitative real-time PCR (qRT-PCR) using the cDNA obtained from the dissected tissues and isoform-specific primers. Primers were designed with Primer Express software (primer sequences in Table 1; Applied Biosystems, Foster City, CA). qRT-PCR was performed in duplicate on a ABI Prism 7000 sequence analysis system (Applied Biosystems), and all reactions contained 2 μl of cDNA, 4 pmol of each primer, double-processed tissue culture water (Sigma-Aldrich), and Universal SYBR green master mix (Applied Biosystems) in a total volume of 22 μl. All qRT-PCR reactions were performed as follows: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Melt curve analysis was performed after each reaction to confirm the presence of only a single product of the reaction. Negative control reactions were performed for all samples with RNA that had not been reverse transcribed to control for the possible presence of genomic DNA contamination. Genomic contamination was <1 in 99 starting cDNA copies for all templates. Heart cDNA was used to develop a standard curve relating threshold cycle to cDNA amount for each primer set. All results were expressed relative to these heart standard curves and normalized to mRNA expression of elongation factor-1α (EF-1α)

Table 1. *PCR primers used for identification and quantification of CPT-1, PDK-2, PPARα, and EF-1α mRNA from tilapia tissues*

Name	Purpose	Direction	Sequence
CPT-1	Degenerate	Forward	5′-GA(C/T) TGG TGG GA(A/G) GA(A/G) TA(C/T) (A/G/T)TC TA-3′
CPT-1	Degenerate	Reverse	5′-GCC CA(A/C/G) GA(A/G) TG(C/T) TC(A/C/T) GC(A/G) TT-3′
PDK-2	Degenerate	Forward	5′-GGA AC(A/C) G(A/C/G)C ACA A(C/T)G A(C/T)G T-3′
PDK-2	Degenerate	Reverse	5′-GT(C/G) CC(A/G) (A/T)A(A/G) CCC TCC AT(A/C/G/T) G-3′
PPARα	Degenerate	Forward	5′-GAG GGC TGC AAG GGT TTC-3′
PPARα	Degenerate	Reverse	5′-CGG AGG TC(A/G) GCC AGT TTC T-3′
CPT_{Til}−1 iso1	qRT-PCR	Forward	5′-GCC GCC TTC TTT GTG ACA CT-3′
CPT_{Til}−1 iso1	qRT-PCR	Reverse	5′-TCT AAA CTG GCT GCT GGG TCA T-3′
CPT_{Til}−1 iso2	qRT-PCR	Forward	5′-CAA ACC TCT CAT GCT CCT ACA CA-3′
CPT_{Til}−1 iso2	qRT-PCR	Reverse	5′-ACA CGA GTC GTG TTG AAC ATA CG-3′
PDK_{Til}−2	qRT-PCR	Forward	5′-CCG CGT AGA CAA TGG TCG TA-3′
PDK_{Til}−2	qRT-PCR	Reverse	5′-GAA ATG GGC AGG CCA TAG C-3′
$PPAR_{Til}$α	qRT-PCR	Forward	5′-CAC GAC ATG GAG ACG TTC CA-3′
$PPAR_{Til}$α	qRT-PCR	Reverse	5′-TCC GGA TAG TCG CTG TTT ATC A-3′
EF-1α	qRT-PCR	Forward	5′-TGA CTG CGC TGT GCT GAT C-3′
EF-1α	qRT-PCR	Reverse	5′-CTT GGA GAT ACC AGC CTC GAA-3′

CPT-1, carnitine palmitoyltransferase-1; PDK-2, pyruvate dehydrogenase kinase-2; PPARα, peroxisome proliferator-activated receptor α; EF-1α, elongation factor-1α; qRT-PCR, quantitative real-time PCR.

(primers in Table 1; designed from the known *O. niloticus* sequence, accession no. AB075952.1). Tissue distributions were similar when expressed relative to total RNA or EF-1α (data not shown).

Hypoxia exposure. Tilapia were fasted for 2 days, and then equal numbers (total = 68, 100–400 g) were transferred to two separate 340-liter aerated aquaria fed with recirculating filtered water at 22°C. Each fish was housed in a separate plastic container within the aquaria. Each container had a mesh top and elongated perforations on each side to allow adequate water flow. The plastic containers were stacked on top of one another and weighted inside with a few clean stones. To ensure good water flow several submersible aquarium pumps were placed at strategic positions in each of the tanks, and a large air stone was present on either end of each tank.

The fish were allowed to acclimate for 24 h. Then, fish comprising the normoxic control group (~92% air saturation) were gently removed from the aquaria in their individual containers in opaque plastic bags filled with the aquarium water. Four fish were removed from each aquarium, for a total of eight fish. A benzocaine solution (100 g/l ethanol) was added to the bags to terminally anesthetize the fish (<30 s). Fish remained calm during this procedure. Immediately after loss of equilibrium, blood was sampled via caudal puncture and placed on ice until analysis of hematocrit (Hct) and hemoglobin (Hb). After blood sampling, the heart was quickly removed, emptied of blood, blotted dry, and frozen in liquid N_2. Plasma was obtained by centrifuging whole blood (3000 *g*; 5 min) and then freezing in liquid N_2. All samples were stored at −80°C until analyses.

After the sampling of the normoxic fish, hypoxia was induced by bubbling N_2 into each aquarium, which was covered with plastic bubble wrap and polystyrene foam to prevent oxygen ingress. Water P_{O_2} decreased with N_2 bubbling from normoxia (~92% air saturation) to 5% air saturation (8 Torr) over a 1.5-h period and was maintained at this level for a 24-h period with dissolved oxygen controllers (alpha-DO2000W, Eutech Instruments, Singapore) connected to solenoid valves that controlled the flow of N_2 into the aquaria. This severe level of hypoxia matched the level of hypoxia used in experimental *series II*. Water P_{O_2} in both tanks was held at the same level and constantly monitored. With the protocol described above, four fish were sampled from each tank (total = 8 fish) after 1, 2, 4, 8, 12, and 24 h of the hypoxia exposure. After the 24-h hypoxia exposure, N_2 bubbling was ceased and the aquarium water was aerated, resulting in a rapid return to normoxia within 1 h. Twelve hours after the return to normoxia, three fish from each aquarium were sampled as above and are termed the recovery group (total = 6 fish).

Analytical protocols. Blood [Hb] was measured spectrophotometrically (45). Hct was determined by centrifugation at 5,000 *g* in a sealed hematocrit capillary tube. Mean cellular Hb content (MCHC) was calculated as [Hb]/Hct. Plasma [lactate] and [glucose] were measured on deproteinized and nondeproteinized plasma, respectively, with the spectrophotometric methods described by Bergmeyer (5). Plasma [NEFA] was measured spectrophotometrically with a commercially available kit [NEFA-HR(2); Wako, Osaka, Japan].

Frozen heart was broken into small pieces under liquid N_2 with an insulated mortar and pestle. Ground tissue was aliquoted into preweighed 1.5-ml microcentrifuge tubes and stored at −80°C until analysis. For the extraction of metabolites, 1 ml of ice-cold 1 M $HClO_4$ was added to a microcentrifuge tube containing tissue and the mixture was immediately sonicated on ice for 20 s with a Kontes sonicator set to its highest setting. The homogenates were centrifuged at 10,000 *g* for 10 min at 4°C, and the supernatant was neutralized with 3 M K_2CO_3. The neutralized extracts were assayed spectrophotometrically for concentrations of ATP, CrP, creatine (Cr), and lactate according to methods described by Bergmeyer (5). Levels of acetyl CoA and acetyl- and free carnitine were assayed radiometrically on neutralized extracts with the methods of Cederblad et al. (9). Acetyl carnitine measurements were corrected for endogenous acetyl CoA. Intracellular pH (pH_i) was measured on a separate aliquot of heart tissue with the methods of Pörtner et al. (39) and a thermostatted (22°C) Radiometer BMS3 Mk2 capillary microelectrode with a PHM84 pH meter (Radiometer, Copenhagen, Denmark).

Expression of mRNA in heart was ascertained by qRT-PCR (see Table 1 for qRT-PCR primer sequences). With the methods outlined above, total RNA was extracted from heart tissue, first-strand cDNA was synthesized from 5 μg of total RNA, and qRT-PCR was performed in duplicate. One randomly selected control sample was used to create a standard curve relating threshold cycle to cDNA amount for each primer set. All results are expressed relative to these standard curves, and mRNA amounts are normalized relative to EF-1α mRNA expression. Real-time primers for EF-1α were designed with a GenBank sequence from *Oreochromis niloticus* (accession no. AB075952.1). There was no effect of hypoxia exposure on the mRNA expression level of EF-1α (data not shown). Expression of mRNA was further normalized by setting the expression in normoxia to 1.

The active fraction of PDH (PDH_a) was measured at 22°C in heart homogenates with the methods described by Richards et al. (42), except that reaction aliquots were sampled at 1, 2, and 3 min because of the relatively high PDH_a activity in heart.

Total PDK-2 protein content was measured by immunoblot analysis according to the protocols described in Richards et al. (45), with minor modifications. Briefly, aliquots of the homogenates used for measurement of PDH_a were frozen at −80°C, thawed, and centrifuged at 1,000 *g* for 10 min at 4°C. The supernatant was diluted to 1 mg protein/ml, denatured by boiling for 3 min in Laemmli buffer, and loaded on denaturing SDS-polyacrylamide gels at a final concentration of 20 μg protein/lane. An identical control sample was included on each gel to control for gel-to-gel variation. Gel electrophoresis and protein transfer to nitrocellulose membranes were carried out as described by Richards et al. (45). Blots were incubated overnight at 4°C with 1 μg/ml of primary antibody (raised against the COOH-terminal end of rabbit PDK-2, peptide sequence 5′-VPSTEPKNT-STYRVS-′3; Abgent, San Diego, CA), followed by incubation for 1 h with alkaline phosphatase-conjugated goat anti-rabbit IgG secondary antibody (Sigma-Aldrich). Blots were developed and the band intensity measured as described by Richards et al. (45). Individual samples were expressed relative to total protein and the gel-to-gel standard and then normalized to the mean of the normoxia control samples. A standard curve of select samples was run to confirm the binding efficiency of the antibody (not shown).

AMPK activity was measured by the modified SAMS peptide [^{32}P]ATP method described by Jibb and Richards (28).

Calculations of energetic status. Measured values of [ATP], [CrP], [Cr] and pH_i were used to calculate free cytosolic [ADP] and [AMP], assuming equilibrium of the creatine kinase and adenylate kinase reactions and following the methods described by Jibb and Richards (28).

Statistical Analyses

Changes in $\dot{M}_{O_2}$ or cardiovascular parameters in *series I* and *II* were tested for statistical significance with a one-way repeated-measures ANOVA followed by the Holm-Sidak post hoc test or, when data were nonnormal and log transformation did not improve normality, a one-way repeated-measures ANOVA on ranks followed by Student-Newman-Keuls (SNK) post hoc test. Comparison of critical P_{O_2} of cardiovascular parameters and $\dot{M}_{O_2}$ was carried out with a one-way ANOVA on ranks followed by SNK post hoc test. Data for *series III* were tested for statistical significance with a one-way ANOVA followed by the Holm-Sidak post hoc test. Statistical significance was accepted at $P < 0.05$. All mean data are presented as means ± SE.

RESULTS

Series I. Assessment of Hypoxia Tolerance

Tilapia showed a typical relationship between $\dot{M}_{O_2}$ and water P_{O_2}. A zone of oxygen-independent $\dot{M}_{O_2}$ occurred be-

tween ≥92% air saturation and the P_{crit} of 18.0 ± 1.5% air saturation (28.5 ± 2.4 Torr), below which $\dot{M}O_2$ became oxygen dependent and decreased with decreasing water P_{O_2} (Fig. 1). The whole blood hemoglobin-oxygen P_{50} was 7.0 ± 0.5% air saturation (10.9 ± 0.8 Torr).

Series II. Cardiac ATP Demand

Our experimental protocol allowed for the simultaneous recording of whole animal $\dot{M}O_2$ and cardiovascular function during progressive depletion of oxygen from 80% to 2.5% air saturation; only cardiovascular function was followed during a subsequent 8-h exposure to hypoxia (5% air saturation) and 1 h of normoxic recovery. Whole animal $\dot{M}O_2$ began to decrease significantly at 20% air saturation, reaching a minimum value at 2.5% air saturation that represented a depression of $\dot{M}O_2$ of >90% compared with normoxic $\dot{M}O_2$ (Fig. 1). P_{crit} was 17.9 ± 1.7% air saturation (28.2 ± 2.6 Torr), which was not significantly different from the P_{crit} determined in *series I*, even though routine normoxic $\dot{M}O_2$ was lower in *series II* relative to *series I* fish (Fig. 1). The lower $\dot{M}O_2$ in *series II* is probably due to the larger size of these fish.

f_H was unaffected by water P_{O_2} between 93% to 30% air saturation. At 20% air saturation there was a significant bradycardia, and below this threshold f_H decreased steadily as water P_{O_2} decreased, reaching a minimum f_H at 2.5% air saturation that was ~45–50% of the normoxic value (≥30% air saturation) (Fig. 2*A*). Bradycardia persisted throughout a subsequent 8-h hypoxia exposure at 5% air saturation (Fig. 2, *A* and *B*). After 1 h of recovery, f_H was significantly elevated above the normoxic values (cf. Fig. 2, *A* and *B*).

The pattern of changes in Q closely paralleled those for f_H. Q was unaffected by decreases in water P_{O_2} from 93% to 20% air saturation but decreased significantly at ≤15% air saturation (Fig. 2*C*). At 5% and 2.5% air saturation, Q had decreased to a minimum value that was 40–45% of that seen in normoxia (≥30% air saturation). During 8-h exposure at 5% air saturation, Q increased slightly and remained at ~55% of the normoxic value (Fig. 2*D*). After 1 h of recovery, Q was significantly elevated above the normoxic value (cf. Fig. 2, *C* and *D*).

The pattern of changes in CPO paralleled those for Q and f_H. CPO was constant at 1.30 mW/g between 93% and 20% air saturation and then decreased significantly at and below 15% air saturation, reaching a minimum value of ~0.5 mW/g at 5% and 2.5% air saturation (Fig. 2*E*). CPO remained depressed at ~0.7 mW/g throughout the 8-h hypoxia exposure at 5% air saturation (Fig. 2*F*). After 1 h of recovery, CPO returned to values that were similar to those seen during normoxia (cf. Fig. 2, *E* and *F*).

V_{SH} remained constant at all oxygen tensions and throughout the 8-h hypoxia exposure (Fig. 3, *A* and *B*). After 1 h of recovery, V_{SH} was significantly elevated compared with the normoxic value (cf. Fig. 3, *A* and *B*). P_{VA} was largely unaffected by decreasing water P_{O_2} but decreased significantly in recovery (Fig. 3, *C* and *D*).

R was unaffected by decreasing water P_{O_2} until 15% air saturation, below which *R* steadily increased to a value at 2.5% air saturation that was approximately double the normoxic level. *R* remained significantly higher than the normoxic level throughout the 8-h hypoxia exposure at 5% air saturation and then decreased significantly after 1 h of recovery (Fig. 3*F*).

Critical P_{O_2} values for all the cardiovascular variables were within a 10% range of air saturation: Q = 25.5 ± 2.3% air saturation (40.3 ± 3.6 Torr); CPO = 23.0 ± 2.9% air saturation (36.3 ± 4.5 Torr); f_H = 23.8 ± 1.3% air saturation (37.6 ± 2.0 Torr); and *R* = 17.1 ± 1.9% air saturation (27.0 ± 3.0 Torr). The critical P_{O_2} for Q, CPO, and f_H were not statistically different from one another, but all were significantly higher than the critical P_{O_2} for *R* ($P < 0.05$). Critical P_{O_2} of $\dot{M}O_2$ (P_{crit}) was significantly lower than the critical P_{O_2} for f_H, Q, and CPO ($P < 0.05$) but was not different from that for *R* ($P > 0.05$).

Series III. Cardiac ATP Supply

Gene identification, sequencing, and tissue distribution. Partial cDNA sequences coding for PPARα ($PPAR_{Til}\alpha$), PDK-2 ($PDK_{Til}-2$) and two isoforms of CPT-1 ($CPT_{Til}-1$ *iso1* and $CPT_{Til}-1$ *iso2*) were identified in tilapia. Alignment of these sequences with those from other vertebrates showed 75–86%, 70–75%, and 56–80% similarity , respectively, to other available isoforms in fishes and mammals. The CPT-1 isoforms showed 66% sequence similarity to one another, and neighbor-joining phylogenetic tree analysis shows that both grouped more closely with the mammalian CPT-1α than with the mammalian CPT-1β isoform (data not shown).

The two isoforms of CPT, $CPT_{Til}-1$ *iso1* and $CPT_{Til}-1$ *iso2*, showed similar tissue mRNA distributions, except that $CPT_{Til}-1$ *iso1* mRNA expression was higher in brain, liver, and red muscle compared with $CPT_{Til}-1$ *iso2*, whereas $CPT_{Til}-1$ *iso2* was higher in white muscle (Fig. 4*A*). mRNA of $PPAR_{Til}\alpha$ was present in all tissues, with the highest levels in heart, red muscle, and brain (Fig. 4*B*). $PDK_{Til}-2$ mRNA was also found in all tissues, with the highest levels in heart and red muscle (Fig. 4*C*). Relative tissue distributions of mRNA expression of the genes were similar whether mRNA was expressed relative to total RNA alone or normalized to EF-1α (data not shown for expression relative to total RNA).

Response to hypoxia exposure. During the first minutes of hypoxia exposure, some of the fish appeared agitated whereas others remained quiescent. By the end of the first 1 h of hypoxia exposure, however, all fish were quiescent and rested quietly on the bottom of their container. Six fish died during the first 8 h of hypoxia exposure.

During hypoxia exposure, Hct did not change markedly from the normoxic value but decreased during recovery (Table 2). Blood [Hb] decreased at 4 h and 8 h of hypoxia exposure as well as during recovery compared with the normoxic value. A decrease in MCHC was observed during hypoxia, followed by a return to normoxic levels during recovery (Table 2).

Plasma [lactate] rapidly increased ~23-fold over the first 8 h of hypoxia exposure and then decreased to a new steady-state level at 12 and 24 h that was significantly elevated above normoxic levels (Fig. 5*A*). Plasma [glucose] increased significantly during the first 12 h of hypoxia exposure and then decreased significantly at 24 h but remained elevated compared with the normoxic value (Fig. 5*B*). Conversely, plasma [NEFA] dropped precipitously during the first 1–2 h of hypoxia exposure and remained depressed for the full 24-h exposure (Fig. 5*C*). [Lactate], [glucose], and [NEFA] in plasma all returned to normoxic levels after 12 h of recovery in normoxia.

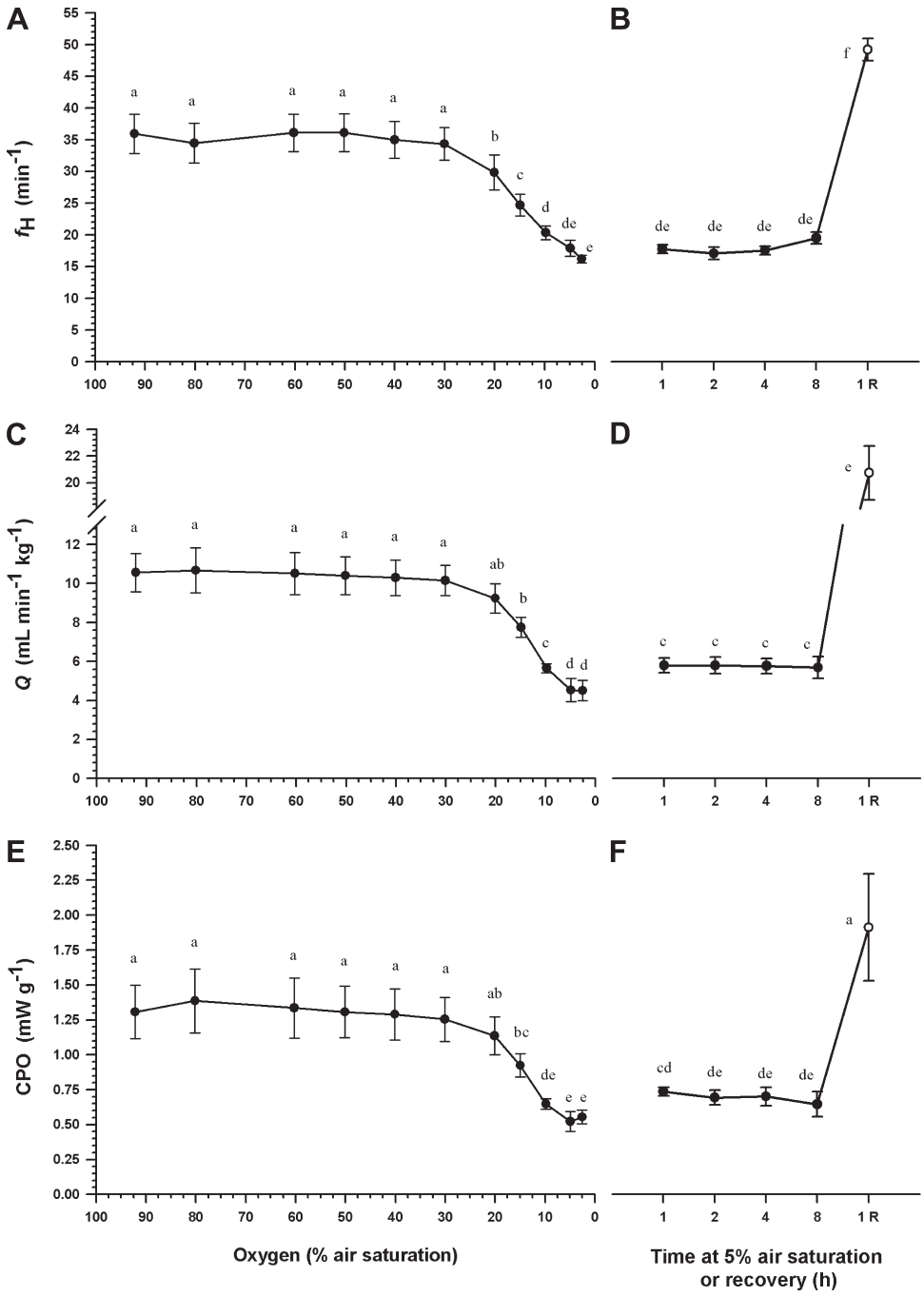

Fig. 2. Heart rate (f_H; *A* and *B*), cardiac output (Q; *C* and *D*), and cardiac power output (CPO; *E* and *F*) of tilapia (*series II*) during progressive decreases in water oxygen content from 92% air saturation to 2.5% air saturation (*A*, *C*, *E*) over 3.6 ± 0.2 h, followed by up to 8 h at 5% air saturation and 1 h of recovery (1 R) in normoxic water (*B*, *D*, *F*). Data are means ± SE ($n = 6$; $n = 5$ for 8 h and recovery points). Values that do not share a letter are significantly different ($P < 0.05$).

Cardiac [ATP] was unaffected by 24 h of hypoxia exposure (Fig. 6*A*). Cardiac [CrP] also did not change markedly during hypoxia exposure but increased after 12-h recovery (Table 3). We were initially concerned by the lack of an effect of hypoxia exposure on cardiac [CrP], so we exposed an additional group of tilapia to 5% air saturation for 2 h and confirmed that cardiac [CrP] was not significantly affected by exposure to this level of hypoxia (B. Speers-Roesch and J. G. Richards, unpublished observation). Free [Cr] in the heart was unaffected by hypoxia exposure and decreased during recovery (Table 3). Calculated [ADP_{free}] and [AMP_{free}] significantly decreased as the hypoxia exposure progressed, reaching a relatively stable level by 8 h that continued in recovery (Table 3). During hypoxia exposure, [lactate] increased significantly in heart and returned to normoxic levels after 12 h of recovery (Fig. 6*B*). Similar to plasma [lactate], heart [lactate] peaked at 8 h of hypoxia exposure and then decreased by 24 h but remained significantly elevated above the normoxic value (Fig. 6*B*). Cardiac pH_i decreased significantly by 0.10–0.15 pH units during hypoxia exposure but returned to normoxic levels during recovery (Fig. 6*C*). Cardiac free [carnitine] and [acetyl carnitine] did not change in any consistent manner during hypoxia; levels stayed roughly the same as those in normoxia throughout the hypoxic and recovery periods (Table 3).

The activity of PDH_a in tilapia heart generally decreased during hypoxia exposure (Fig. 7*A*), with a 30–35% decrease in

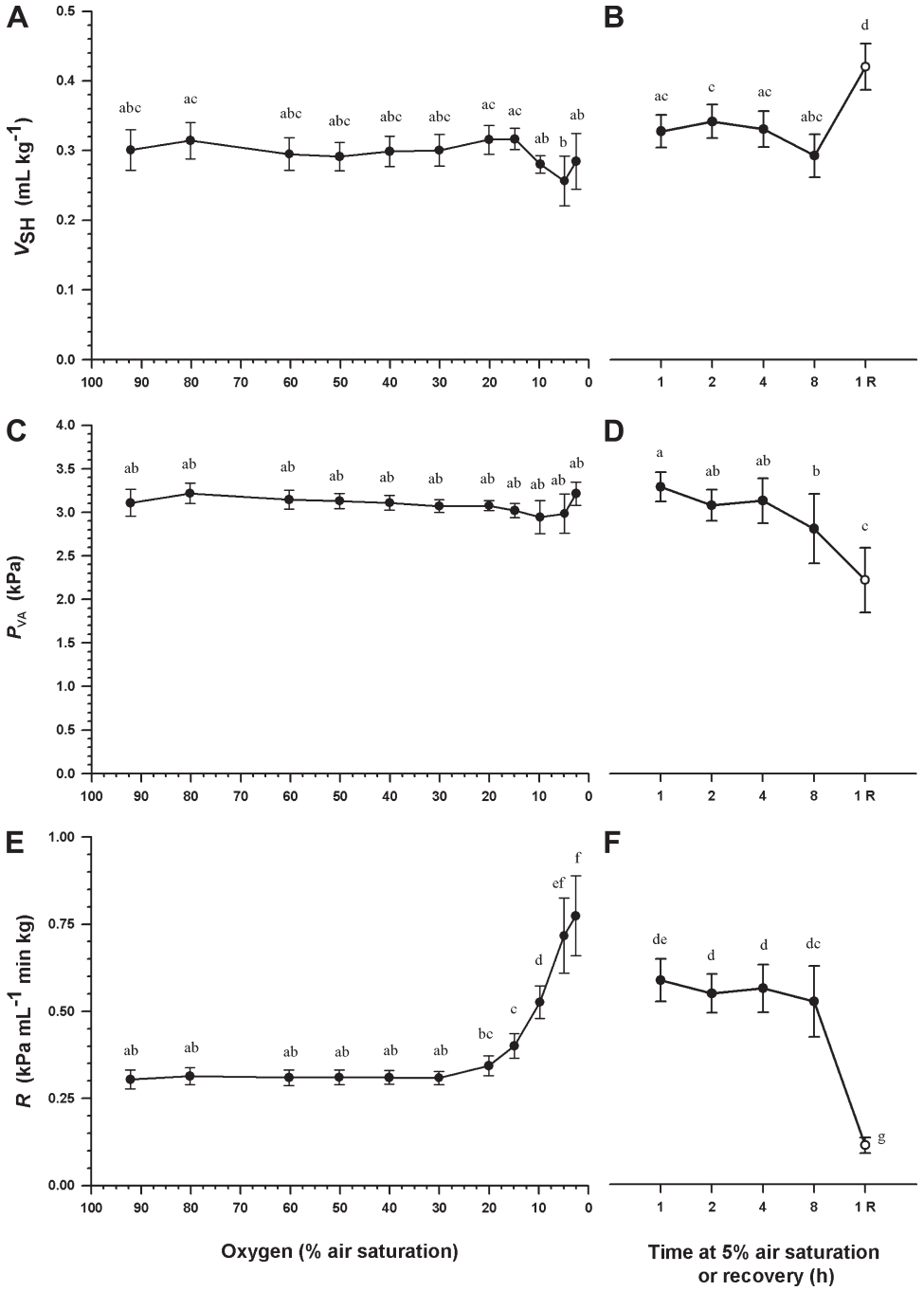

Fig. 3. Stroke volume (V_{SH}; *A* and *B*), ventral aortic pressure (P_{VA}; *C* and *D*), and peripheral resistance (*R*; *E* and *F*) of tilapia (*series II*) during progressive decreases in water oxygen content from 92% air saturation to 2.5% air saturation (*A*, *C*, *E*) over 3.6 ± 0.2 h, followed by up to 8 h at 5% air saturation and 1 h of recovery (1 R) in normoxic water (*B*, *D*, *F*). Data are means ± SE ($n = 6$; $n = 5$ for 8 h and recovery points). Values that do not share a letter are significantly different ($P < 0.05$).

mean activity in the first 4 h and a significant 50–60% depression at 8 h and 24 h. At 12 h, however, PDH_a activity was not significantly different from the normoxic level. During recovery, cardiac PDH_a activity returned to the normoxic level. Heart [acetyl CoA], the product of the reaction catalyzed by PDH as well as of fatty acid oxidation, decreased significantly by 30–40% during the first 1–4 h of hypoxia exposure and remained depressed during recovery (Fig. 7*B*). $PDK_{Til}-2$ transcript levels increased by approximately twofold over the first 8 h of hypoxia exposure, followed by a decrease back to normoxic levels for the remainder of the hypoxia exposure and recovery (Fig. 7*C*). However, the amount of PDK-2 protein in heart of tilapia was unaffected during hypoxia exposure and recovery (Fig. 7*D*).

Levels of mRNA of $CPT_{Til}-1\ iso1$ and $CPT_{Til}-1\ iso2$ were unchanged in heart during hypoxia exposure and recovery (Table 4). The quantity of $PPAR_{Til}\alpha$ transcript increased significantly in the first 8 h of hypoxia, followed by a decline to levels statistically indistinguishable from normoxic levels by 12-h exposure (Table 4).

The activity of AMPK was assessed in heart only during normoxia and at 1 h, 2 h, and 8 h during the hypoxia exposure, but no significant change in activity was observed (Table 5).

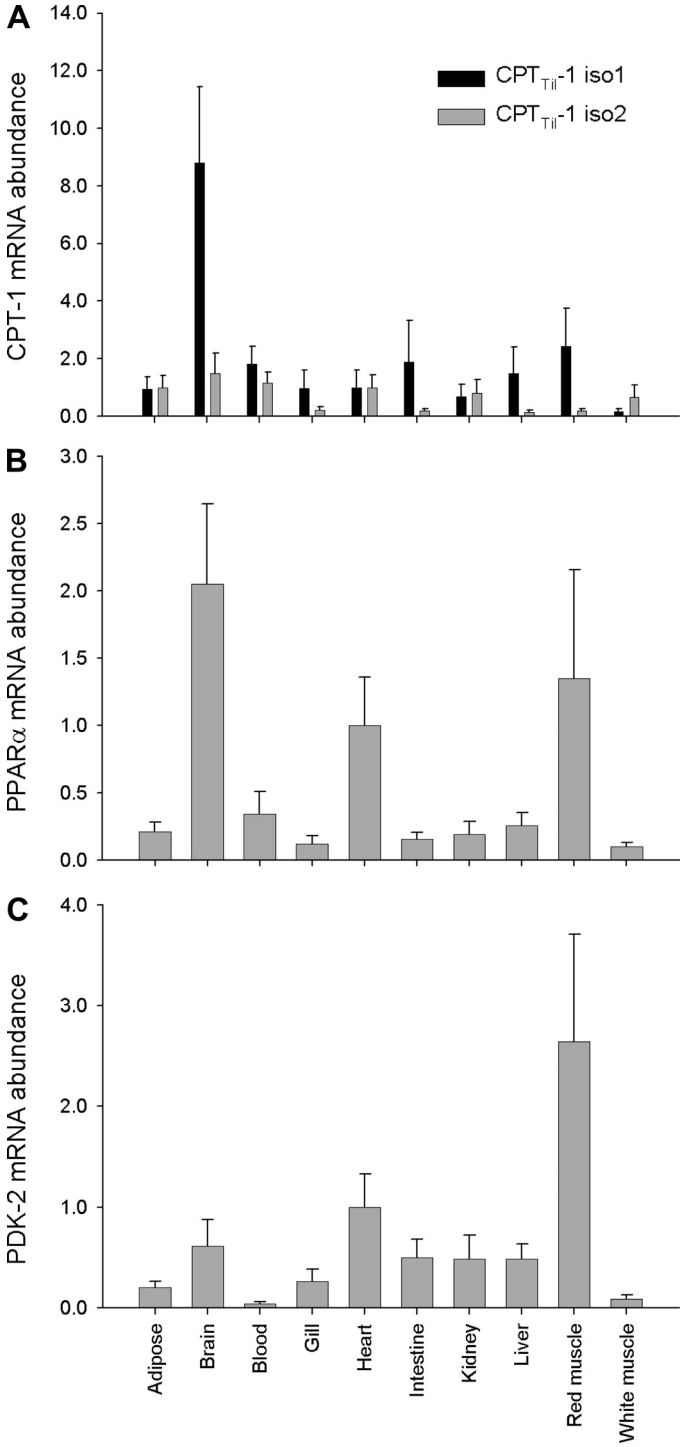

Fig. 4. Relative tissue distribution of mRNA of carnitine palmitoyltransferase ($CPT)_{Til}$*−1 iso1* (*A*), CPT_{Til}*−1 iso2* (*A*), peroxisome proliferator-activated receptor ($PPAR)_{Til}\alpha$ (*B*), and pyruvate dehydrogenase (PDH) kinase ($PDK)_{Til}$*−2* (*C*) genes in tissues from tilapia (means ± SE, $n = 3$). mRNA levels are normalized to elongation factor-1α (*EF-1α*) mRNA expression, with the level in heart set to 1.

DISCUSSION

The ability to suppress cellular and whole animal ATP turnover during oxygen lack is a unifying strategy underlying hypoxia tolerance in many vertebrates (7). In tilapia, exposure to hypoxia is marked by a large and rapid decrease in whole animal $\dot{M}O_2$ (Fig. 1). During exposure to progressive hypoxia, $\dot{M}O_2$ decreased to ~23% and ~7% of normoxic values at 5% and 2.5% air saturation, respectively, which is similar to the 70–80% reduction of $\dot{M}O_2$ at 5% air saturation measured by van Ginneken et al. (54) in Mozambique tilapia (*Oreochromis mossambicus*). In our study, depression of $\dot{M}O_2$ occurred below the tilapia P_{crit} of ~18% air saturation (28 Torr), which is also similar to that recorded previously for Nile tilapia (*O. niloticus*) (11, 59). Although P_{crit} is generally considered to be a useful estimator of hypoxia tolerance (11), tilapia possess a P_{crit} that is similar to that of the intolerant rainbow trout at 20°C and higher than those of other hypoxia-tolerant tropical cichlids, which range from 8 to 16 Torr (5%-10% air saturation) at 20°C (11, 37). However, a low whole blood hemoglobin-oxygen P_{50} in tilapia (7% air saturation or 10.9 Torr in whole blood with pH 7.7–7.8 at 22°C), about half that seen in rainbow trout under similar conditions (36), would facilitate oxygen extraction from the environment during exposure to hypoxia.

In most vertebrates, the heart is exquisitely sensitive to oxygen lack and susceptible to necrosis because of an inability to match ATP supply and demand. The maintenance of stable [ATP] in tilapia hearts during exposure to severe hypoxia (Fig. 6*A*) represents a hallmark measure of hypoxia tolerance (7) and is achieved through a combination of increased reliance on oxygen-independent ATP product via anaerobic glycolysis (Figs. 5*A* and 6*B*) and a rapid, large, and sustained reduction in CPO (Fig. 2, *E* and *F*). Depression of CPO reduces cardiac ATP demand to levels that can be sustained by anaerobic glycolysis alone (20, 38). The ability of hypoxia-tolerant fishes such as tilapia to modulate CPO during periods of low oxygen is therefore likely important in ensuring whole organism hypoxia survival.

Cardiac ATP Demand

Few studies have examined how the cardiovascular status of hypoxia-tolerant fishes responds to progressive decreases in water P_{O_2}. During progressive decreases in environmental oxygen, we show that all cardiovascular parameters in tilapia remain at resting normoxic levels down to a water P_{O_2} of 30% air saturation (Fig. 2, *A*, *C*, *E*, and Fig. 3, *A*, *C*, *E*). Our normoxic values compare well with previous studies. For example, f_H is virtually identical to that measured in Nile tilapia by Thomaz et al. (53), and resting values for P_{VA} and CPO fall within the normal range observed in teleosts (2.9–4.9 kPa and 0.5–3.0 mW/g, respectively) (50). At and below 20% air saturation a substantial bradycardia develops as $\dot{M}O_2$ falls, and the bradycardia is sustained at ~50% of normoxic f_H for 8 h of hypoxia exposure at 5% air saturation (Fig. 2, *A* and *B*). Hypoxic bradycardia is observed in many, but not all, fishes and generally is mediated by cholinergic input via vagal innervation from gill chemosensors (21). Direct depressive effects of hypoxemia on f_H also occur (21, 35). The magnitude of bradycardia depends on numerous factors including temperature and the depth of hypoxia, but a halving of f_H is not atypical in severe hypoxia exposure (19, 21). As seen in tilapia, development of bradycardia during progressive hypoxia below a certain, species-specific P_{O_2} is observed in many teleosts [e.g., lingcod (*Ophiodon elongatus*) (18), *Hoplias* spp. (40),

Table 2. *Blood hemoglobin, hematocrit, and mean cellular hemoglobin content in tilapia exposed to normoxia or ≤24-h severe hypoxia (5% air saturation) and after 12-h recovery in normoxic water*

	Normoxia	Hypoxia						24-h Hypoxia +12-h Recovery
		1 h	2 h	4 h	8 h	12 h	24 h	
Hb, mM	1.30±0.04[a,c]	1.20±0.05[a,c]	1.12±0.07[a,b]	1.00±0.10[b]	0.96±0.06[b]	1.21±0.07[a,c]	1.31±0.07[c]	0.98±0.04[b]
Hct, %	31.5±0.8[a]	33.6±0.9[a]	32.3±1.0[a]	30.9±1.5[a]	26.9±1.2[b]	31.5±1.4[a]	34.1±1.2[a]	24.0±1.3[b]
MCHC, [Hb]/Hct	4.12±0.07[a,d]	3.56±0.12[b,c]	3.45±0.12[b,c]	3.19±0.21[c]	3.57±0.09[b,c]	3.90±0.31[a,b,d]	3.83±0.15[a,b]	4.35±0.12[d]

Data are means ± SE ($n = 8$, except $n = 6$ for 12-h recovery). Hb, hemoglobin; Hct, hematocrit; MCHC, mean cellular Hb content. Values with different superscripted letters are significantly different ($P < 0.05$, 1-way ANOVA with Holm-Sidak test).

Atlantic cod (*Gadus morhua*) (21), Japanese eel (*Anguilla japonica*) (10)] and may be related to each species' P_{crit} and the depression of $\dot{M}O_2$. In fact, P_{crit} matches the P_{O_2} at initiation of bradycardia (i.e., P_{crit} of f_H) in two *Hoplias* spp. (40), the spangled perch (*Leiopotherapon unicolor*) (22), and cod (35), and parallel decreases in f_H and $\dot{M}O_2$ below P_{crit} were observed in these species. Our measurements in tilapia show a similar result. The calculated critical P_{O_2} for f_H was only slightly higher than for $\dot{M}O_2$, and this appears to be explained by a steeper slope of metabolic rate depression compared with bradycardia as hypoxia progressed rather than by an intrinsically different inflection point between these parameters (cf. Fig. 1 and Fig. 2*A*). Instead, the observation of initiation of bradycardia (i.e., first significant decrease in f_H) at the same P_{O_2} (20% air saturation) as $\dot{M}O_2$ first decreased significantly from normoxic values supports the contention that these events occur more or less simultaneously in response to hypoxia. This may in part be due to neural and humoral mechanisms responding to similar input from distinct or shared oxygen chemosensors. The concurrent decreases of $\dot{M}O_2$ and bradycardia as metabolic rate depression progresses may be similarly explained. Supporting a role for simultaneous regulation of $\dot{M}O_2$ and f_H by distinct oxygen sensors, vagotomy and the resulting abolishment of reflex bradycardia due to gill oxygen sensing in cod decreased the P_{crit} of f_H to below the unchanged P_{crit} of $\dot{M}O_2$, whereas in sham-operated cod these values were the same (35). Reduced tissue oxygen demand caused by tissue-level metabolic rate depression also may influence the decreases in f_H (and Q; see below) via neural and humoral mechanisms. A close association between $\dot{M}O_2$, Q, and f_H during routine and elevated activity is well established for fishes and largely reflects changes in tissue oxygen demand (62). Although vagotomy and delay of bradycardia onset had no effect on responses of $\dot{M}O_2$ during graded hypoxia in cod (35), Q was not measured and further studies are needed on the effect of pharmacologically manipulating f_H and other cardiovascular parameters on the $\dot{M}O_2$ of fishes during progressive hypoxia.

Because of the lack of a significant compensatory increase in V_{SH} (Fig. 3, *A* and *B*), the observed bradycardia caused a substantial (~50%) decrease in Q that also developed as $\dot{M}O_2$ fell and was sustained during 8 h of hypoxia (Fig. 2, *C* and *D*). This finding contrasts with the typical observation of increases in V_{SH} in fishes during hypoxia exposure that help maintain Q at least until a certain level of hypoxia is reached (21). However, in several fishes, including short-horn sculpin (*Myoxocephalus scorpius*) (31), common carp (50), lingcod (18), and Japanese eel (10), modest (or absent in the case of the short-horn sculpin) increases in V_{SH} coupled with large decreases in f_H led to a reduction in Q during hypoxia, similar to what we observed in tilapia. Direct comparisons across studies and species are complicated because of differences in the severity of hypoxia used in each study, the usage of graded vs. steady-state hypoxia, and the uncertain relative hypoxia tolerance of the species investigated. More clearly than in previous studies, we show that in the hypoxia-tolerant tilapia Q is rapidly decreased via bradycardia (in the absence of compensatory changes in V_{SH}) as hypoxia and metabolic rate depression develops below P_{crit}, and this depression is sustained throughout the hypoxic exposure.

The reductions of f_H and Q benefit cardiac hypoxia tolerance in tilapia because they facilitate the rapid decrease of CPO and thus ATP demand of the heart to below the level that can be sustained by maximum anaerobic glycolytic flux alone (suggested to be ~0.7 mW/g at 15°C in ectotherms; Refs. 19, 20). This also appears to be the case for common carp, in which the heart operates above this estimated CPO threshold in normoxia, but during oxygen deprivation CPO falls well below this value (50). Conversely, the crucian carp maintains routine CPO low enough that further downregulation is unnecessary during hypoxia (51). Assuming that cardiac glycolytic capacity scales with temperature with a Q_{10} of 2 (38), then the estimated CPO sustainable by glycolysis alone at 22°C for tilapia is ~1.1 mW/g. Like the common carp, tilapia heart in normoxia operated slightly above this level (~1.3 mW/g, which is considerably lower than the maximum aerobic CPO of ~3.8 mW/g observed after exhaustive exercise; B. Speers-Roesch and E. Sandblom, unpublished observation). Then, as hypoxia increased, CPO of tilapia heart decreased in the same fashion as f_H and Q and remained at ~50% of the resting value for the duration of the hypoxic exposure (Fig. 2, *E* and *F*), well below the estimated CPO sustainable by maximum glycolytic capacity. Unlike cold anoxic turtles, but as in common carp, the depression is not great enough to prevent activation of a Pasteur effect (20), and there was a rapid accumulation of lactate in tilapia during a similar hypoxia exposure (Fig. 6*B*). To our knowledge, our measurements of CPO in tilapia are the first published for fish exposed to graded hypoxia and demonstrate that in a hypoxia-tolerant teleost ATP demand of the heart is rapidly downregulated as oxygen levels fall below P_{crit} and whole animal metabolic rate depression develops. In hypoxic common carp, reductions in CPO appear to be caused by decreases in both Q and P_{VA} (50). In tilapia, however, P_{VA} was unchanged (Fig. 3, *C* and *D*), and thus arterial hypotension did not contribute to reduced CPO. Instead, reductions in f_H and Q appear to be the main determinants of decreased CPO in tilapia heart during hypoxia exposure, supporting the argument that a major benefit of hypoxia-induced bradycardia in fishes is to

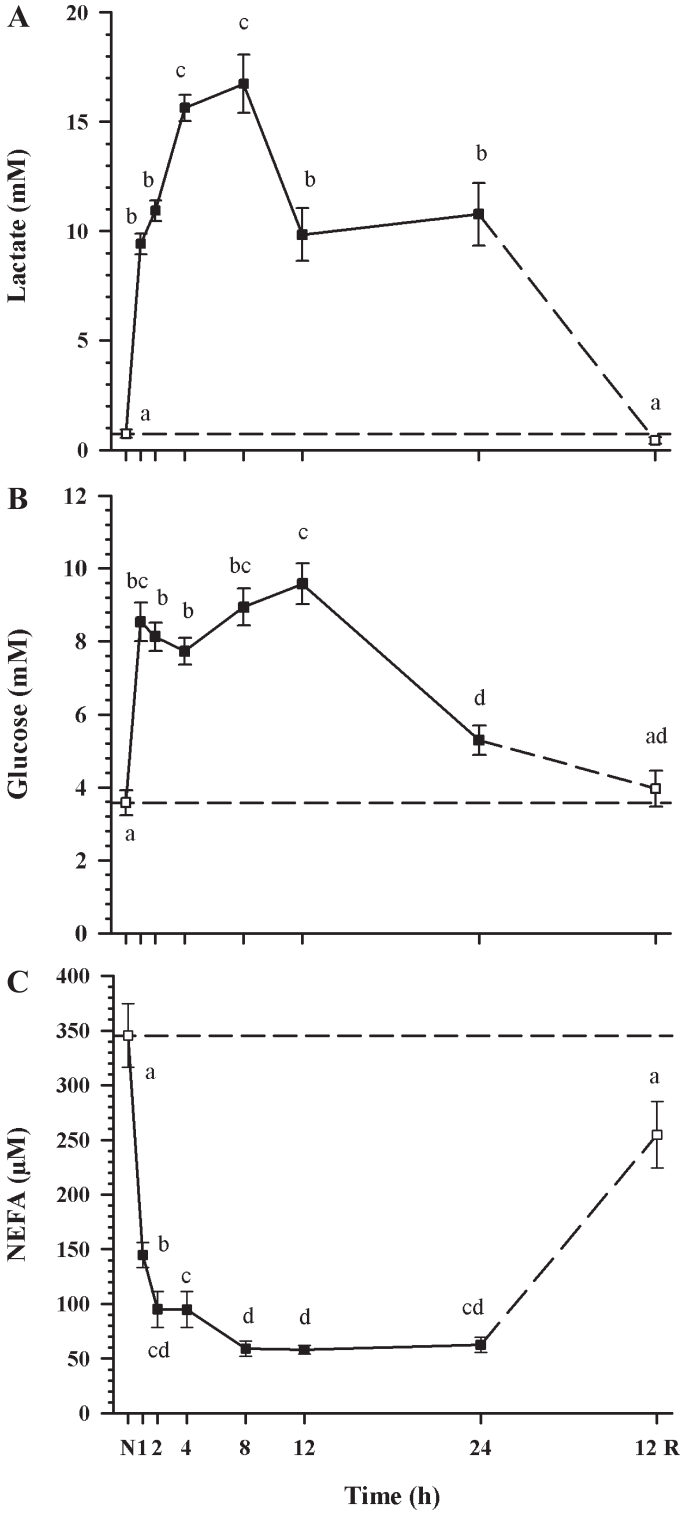

Fig. 5. Plasma [lactate] (*A*), [glucose] (*B*), and [nonesterified fatty acids (NEFA)] (*C*) in tilapia exposed to normoxia (N) or up to 24-h severe hypoxia (5% air saturation) and after 12 h of recovery (12 R) in normoxic water. Data are means ± SE (n = 6–8). Values that do not share a letter are significantly different ($P < 0.05$).

reduce ATP demand of the heart and depress cardiac ATP turnover (19).

It has been suggested that a resetting of the barostatic reflex to facilitate arterial hypotension is a common response to exposure to low oxygen in hypoxia-tolerant but not hypoxia-sensitive teleosts (50). Our results for the hypoxia-tolerant tilapia do not provide support for this hypothesis. Instead, it appears that in tilapia an elevation of R in hypoxia, which occurs in many fishes including common carp (50), helps conserve arterial blood pressure at normoxic levels in the face of large reductions in Q (Fig. 3, *E* and *F*). The increased R probably reflects a peripheral vasoconstriction that shunts

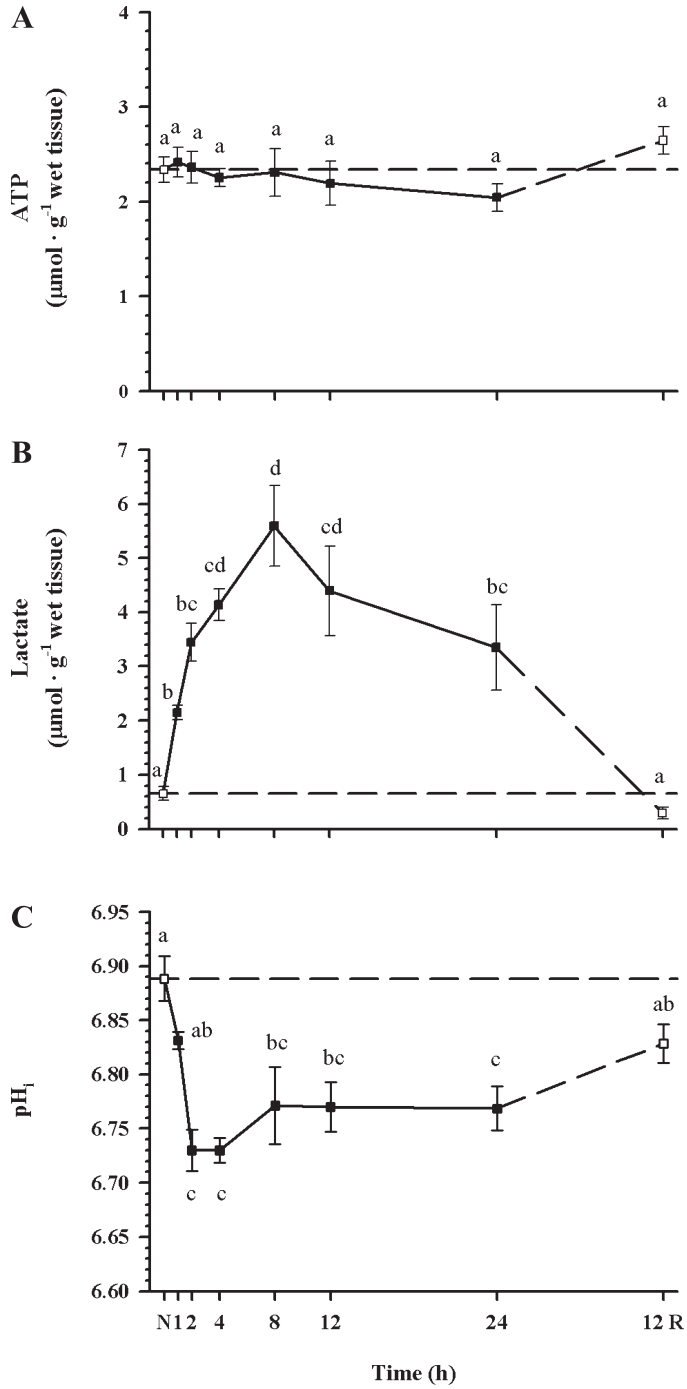

Fig. 6. Heart [ATP] (*A*), [lactate] (*B*), and intracellular pH (pH_i; *C*) in tilapia exposed to normoxia (N) or up to 24-h severe hypoxia (5% air saturation) and after 12 h of recovery (12 R) in normoxic water. Data are means ± SE (n = 5–8). Values that do not share a letter are significantly different ($P < 0.05$).

Table 3. *Heart [creatine phosphate], free [creatine], [ADP_{free}], [AMP_{free}], free [carnitine], and [acetyl carnitine] in tilapia exposed to normoxia or ≤24-h severe hypoxia (5% air saturation) and after 12-h recovery in normoxic water*

	Normoxia	Hypoxia						24-h Hypoxia +12-h recovery
		1 h	2 h	4 h	8 h	12 h	24 h	
[CrP]	1.62 ± 0.28^{a}	$1.94\pm0.24^{a,b}$	1.50 ± 0.16^{a}	$1.94\pm0.31^{a,b}$	2.74 ± 0.48^{b}	$2.49\pm0.60^{a,b}$	$2.24\pm0.34^{a,b}$	3.87 ± 0.37^{c}
[Cr]	$1.86\pm0.16^{a,b,c}$	2.09 ± 0.15^{a}	$1.92\pm0.08^{a,b}$	$1.53\pm0.13^{b,c}$	$1.50\pm0.17^{b,c}$	$1.81\pm0.23^{a,b,c}$	$1.37\pm0.21^{c,d}$	0.93 ± 0.25^{d}
[ADP_{free}]	12.3 ± 1.8^{a}	10.9 ± 1.8^{a}	$9.67\pm0.63^{a,b}$	$6.50\pm1.28^{b,c,d}$	$4.33\pm0.71^{c,d}$	$7.14\pm1.46^{b,c}$	3.62 ± 0.32^{d}	2.46 ± 0.82^{d}
[AMP_{free}]	0.08 ± 0.03^{a}	$0.06\pm0.02^{a,b}$	$0.04\pm0.01^{a,b}$	$0.03\pm0.01^{b,c}$	0.01 ± 0.002^{c}	$0.03\pm0.01^{a,b,c}$	0.008 ± 0.002^{c}	0.004 ± 0.002^{d}
Free [carnitine]	84.9 ± 13.7^{a}	94.4 ± 15.8^{a}	117.6 ± 8.2^{a}	73.8 ± 9.3^{a}	110.3 ± 19.9^{a}	85.1 ± 10.0^{a}	93.3 ± 12.9^{a}	89.8 ± 22.3^{a}
[Acetyl carnitine]	$42.7\pm5.0^{a,b,c,d,e}$	$48.5\pm11.7^{a,c,e}$	64.8 ± 11.3^{a}	$24.2\pm7.6^{b,d,f}$	$57.6\pm10.0^{a,c,e}$	$38.2\pm2.6^{c,d,e}$	$28.8\pm3.1^{e,f}$	$30.0\pm7.4^{e,f}$

Data (in nmol/g wet tissue, except CrP and Cr, which are in μmol/g wet tissue) are means ± SE (n = 5–8). CrP, creatine phosphate; Cr, free creatine. Values that do not share a superscripted letter are significantly different ($P < 0.05$, 1-way ANOVA with Holm-Sidak test).

blood away from tissues with low oxygen demands in hypoxia such as white muscle and the gastrointestinal tract to those with higher oxygen demands such as the heart and brain (50).

The cardiovascular status in tilapia recovering at 1 h after hypoxia warrants brief mention. The rapid elevation of f_H and V_{SH} and thus Q, as well as a decrease in *R* (Fig. 2, *B* and *D*, and Fig. 3, *B* and *F*), are probably important in recovering the oxygen debt of tissues, restoring acid-base status, and flushing accumulated metabolic wastes. Similar responses are observed in recovery from exhaustive exercise in fishes (see, e.g., Ref. 18). The observation of enhanced cardiovascular status following reoxygenation suggests that the tilapia heart does not sustain any significant irreversible damage or impairment after >8 h of hypoxia exposure.

Cardiac ATP Supply

During exposure to low oxygen, ATP supply in hypoxia-tolerant animals is supported primarily by oxygen-independent ATP production and to a much lesser extent by substrate oxidation (7). In *series III*, we exposed tilapia for 24 h to severe hypoxia (5% air saturated water) representing ~30% and 70% of P_{crit} and hemoglobin-oxygen P_{50}, respectively. Like many fishes, the tilapia ventricle lacks coronary arteries (S. Pieperhoff and A. P. Farrell, unpublished observation), so its oxygen supply is solely from venous blood (21). Hematologic parameters related to oxygen transport were largely unaffected (Table 2), and inadequate oxygen delivery and resulting hypoxemia were evident from the large and rapid increase in plasma [lactate] (Fig. 5*A*). A significant increase in plasma [glucose], often seen in response to hypoxia exposure in fishes (see, e.g., Ref. 15), is thought to reflect mobilization of hepatic glycogen stores to fuel anaerobic glycolysis in other tissues, such as the heart. In fact, heart of tilapia exposed to severe hypoxia accumulated lactate (Fig. 6*B*), probably partly due to an increase in plasma [lactate] but also demonstrating a reliance on anaerobic glycolysis in the heart because blood was blotted from the heart during sampling. In both heart and plasma, [lactate] peaked at 8 h, followed by a significant decrease and stabilization at 12 h and 24 h (cf. Figs. 5*A* and 6*B*). This biphasic response in cardiac [lactate] may indicate that matching of ATP supply with demand and depression of ATP turnover was not optimized until 12–24 h of hypoxia, at which point fully realized metabolic depression also may have allowed for the oxidation of some of the accumulated lactate. Activation of anaerobic glycolysis and associated ATP hydrolysis led to a significant decrease of pH_i in the heart (Fig. 6*C*). Similar reductions in pH_i (0.1–0.2 units) have been recorded from white skeletal and cardiac muscles of oscar and turtles, respectively (45, 61).

There was no indication that CrP was utilized for oxygen-independent ATP production because cardiac [CrP] was not depleted after exposure to hypoxia (Table 3). Maintenance of cardiac [CrP] also has been observed in hearts of flounder (*Platichthys flesus*) and lungfish (*Protopterus aethiopicus*) but not rainbow trout during hypoxia exposure (14, 15, 29) and contrasts with the depletion typically seen in other tissues of fishes during hypoxia, including Nile tilapia (15, 28, 44, 45, 55). It is possible that the protection of [CrP] in heart of hypoxia-tolerant fishes may help maintain contractile function during periods of low oxygen. Depletion of CrP and accumulation of P_i is associated with contractile dysfunction and cardiac failure during hypoxia in both mammals and fish (1, 3).

Stable [CrP] in the heart of hypoxic tilapia also reflects adequate matching of ATP supply with demand, which avoids the need to dephosphorylate CrP to maintain [ATP] (27). Indeed, cardiac [ATP] during the hypoxia exposure was unchanged from that in normoxia (Fig. 6*A*). Maintenance of stable cellular [ATP] in the face of hypoxic stress is considered to be characteristic of hypoxia-tolerant animals (7). Constant cardiac [ATP] has been observed in lungfish, flounder, and European eel (*Anguilla anguilla*) exposed to hypoxia (15, 29, 58), whereas in hypoxia-intolerant rainbow trout, cardiac [ATP] decreases significantly (14). In all of these species [ADP] and [AMP] remained unchanged, but the significance of this is unclear because these values are total (bound and free) adenylates and not the free adenylates that are of regulatory relevance (46). In this study, estimated [ADP_{free}] and [AMP_{free}] decreased over the first few hours of hypoxia exposure, reaching a new, relatively stable level from 8 to 24 h and remaining there in recovery (Table 3). These observations in heart contrast with the increase in [ADP_{free}] and [AMP_{free}] typically seen in white muscle and liver of teleosts exposed to hypoxia (28, 44, 55), and the difference appears mostly to be due to depletion of CrP in white muscle and liver, whereas cardiac [CrP] is maintained. The absence of major perturbation of the energetic status of the heart of hypoxic tilapia (Fig. 6*A*; Table 3) in part may be explained by a rapid decrease in ATP demand and ATP turnover via depressed CPO observed in the heart during hypoxia in *series II* (Fig. 2, *E* and *F*). However, because CPO reaches its minimum well before [ADP_{free}] and [AMP_{free}] stabilize, reductions in other pathways of ATP demand such as protein synthesis may also play a role (30). Longer response

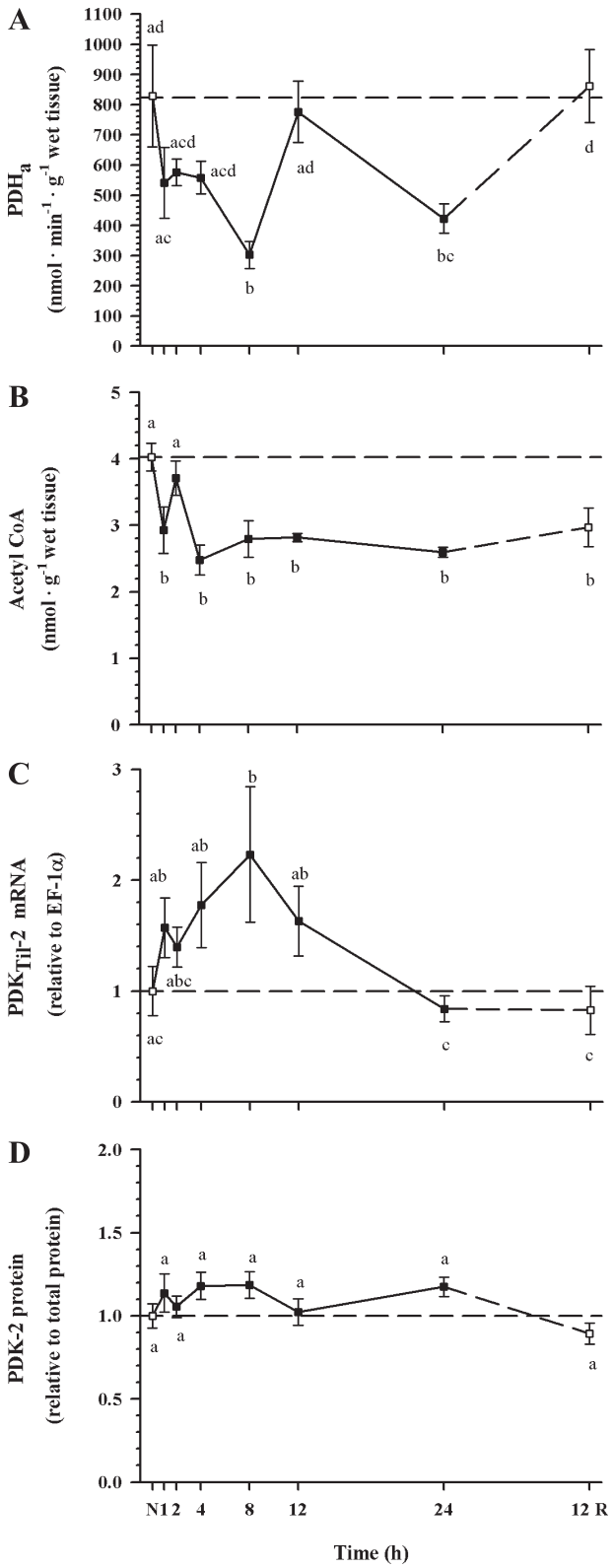

Fig. 7. Heart pyruvate dehydrogenase active form (PDH_a) activity (*A*), [acetyl CoA] (*B*), $PDK_{Til}-2$ mRNA (*C*), and PDK-2 total protein (*D*) in tilapia exposed to normoxia (N) or up to 24-h severe hypoxia (5% air saturation) and after 12 h of recovery (12 R) in normoxic water. Data are means ± SE (n = 6–8). Values that do not share a letter are significantly different ($P < 0.05$).

times for depression of certain ATP demand processes may also explain why [lactate] did not stabilize until 12 h of hypoxia exposure.

Understanding how aerobic ATP supply pathways are controlled in hypoxia-tolerant animals during periods of low oxygen is of interest because low levels of substrate oxidation occur during hypoxia (unlike that expected during anoxia exposure) and potentially deleterious effects of impaired substrate oxidation (e.g., ROS, lipotoxicity) must be avoided. Also, the initiation of metabolic rate depression is thought to be under the control of pathways of aerobic ATP supply, such as mitochondrial substrate oxidation (6). A potential mechanism for this downregulation is the modulation of PDH_a activity. Decreased PDH_a activity has been observed in metabolically depressed snails as well as in white muscle of fishes exposed to hypoxia (8, 44, 45). The results of this study (*series III*) suggest that there is also a general depression of PDH_a activity in the heart of tilapia during hypoxia exposure (Fig. 4*A*). Unlike the more rapid depression seen in white muscle of hypoxic fishes (44, 45), however, a significant decrease in activity is not observed until 8 h of hypoxia exposure, although this partly may be due to the high variation in normoxic fish. Furthermore, the activity decrease is not sustained: at 12 h, activity returned to a level similar to that in normoxia (Fig. 4*C*). Richards et al. (44) observed the same result at 12 h of hypoxia in killifish white muscle, so some temporal fluctuation in PDH activity appears to be normal during hypoxia in fishes.

PDH_a activity is potentially regulated by modulators such as ATP and acetyl CoA acting on existing PDK protein and is influenced by activity of the heart, as well as by regulation of PDK expression (26). Cardiac [acetyl CoA] in tilapia decreased significantly during hypoxia (Fig. 7*B*). Assuming the total CoA pool remained constant, this suggests that activation of PDH_a activity via PDK would be favored. PDH_a activity generally decreased in this study, however, suggesting that significant modulation of PDH activity via acetyl CoA is not occurring. The decrease in acetyl CoA may instead simply reflect a slowing of cellular metabolic rate and the observed reduced workload (Fig. 2, *E* and *F*) of the heart of hypoxia-exposed tilapia. The decrease in cardiac work also may in part explain the decrease in PDH_a activity; in fish skeletal muscle, at least, PDH_a activity is highly responsive to changes in muscular activity (43). The effects on PDH_a activity of manipulation of workload, and thus ATP demand, of isolated hearts operating at different P_{O_2} are needed to assess whether decreases in PDH_a activity in hypoxia are due simply to reductions of CPO or due to active downregulation.

Similar to white muscle of hypoxic killifish (44), we observed a twofold increase in mRNA expression of a PDK-2 isoform ($PDK_{Til}-2$) but no change in PDK-2 total protein in tilapia heart during hypoxia (Fig. 7, *C* and *D*). The tissue distribution of $PDK_{Til}-2$ is comparable to that of a PDK-2 isoform in killifish (44) with relatively high levels in heart (Fig. 4*C*). A disconnect between mRNA and protein levels also has been observed in previous studies on hypoxia exposure in fishes (e.g., Refs. 44, 45). Relative increases of important mRNA transcripts in hypoxia may help ensure that the limited capacity for translation that occurs during hypoxia favors these transcripts to maintain protein level (44). Alternatively, the disconnect may be caused by poor specificity of mammalian antibody for the specific isoform measured via qRT-PCR.

Table 4. *$CPT_{Til}-1$ iso1, $CPT_{Til}-1$ iso2, and $PPAR_{Til}\alpha$ mRNA in heart of tilapia exposed to normoxia or ≤24-h severe hypoxia (5% air saturation) and after 12-h recovery in normoxic water*

	Normoxia	Hypoxia						24-h Hypoxia +12-h Recovery
		1 h	2 h	4 h	8 h	12 h	24 h	
$CPT_{Til}-1$ iso1	1.00±0.25[a]	0.68±0.22[a]	0.70±0.13[a]	0.78±0.27[a]	1.14±0.36[a]	0.60±0.13[a]	0.97±0.28[a]	0.83±0.18[a]
$CPT_{Til}-1$ iso2	1.00±0.8[a]	1.80±0.38[a]	2.81±1.03[a]	1.39±0.41[a]	1.18±0.39[a]	0.53±0.20[a]	0.80±0.09[a]	0.85±0.31[a]
$PPAR_{Til}\alpha$	1.00±0.11[a]	1.48±0.24[a,b]	1.55±0.24[a,b]	2.05±0.27[b]	2.24±0.43[b]	1.26±0.29[a]	1.75±0.23[a,b]	1.38±0.34[a,b]

Data are means ± SE (n = 7 or 8, except n = 5 for 12-h recovery,). mRNA expression is reported relative to the mRNA expression of a control gene (*EF-1α*). Values with different superscripted letters are significantly different ($P < 0.05$, 1-way ANOVA with Holm-Sidak test).

The hypoxic induction of $PDK_{Til}-2$ was transient, decreasing at 12 h and 24 h (Fig. 7*C*). Mitigation of tissue hypoxemia via improved oxygen delivery to the heart is not a likely explanation because lactate load in plasma and heart remained greatly elevated at 12 h and 24 h (Figs. 5*A* and 6*B*). The transient increase in $PDK_{Til}-2$ mRNA instead may reflect an acute response to hypoxia, possibly contributing to downregulation of PDH_a activity (although this remains unconfirmed), which ebbs during acclimation associated with longer-term exposure. Supporting this idea, hearts of zebra fish (*Danio rerio*) exposed to chronic hypoxia (10% air saturation for several weeks) show a greater than threefold decrease of PDK-2 mRNA expression (33). Marques et al. (33) also observed decreases in mRNA expression of genes related to fatty acid oxidation, suggesting a switch to carbohydrate rather than lipid oxidation in heart of zebra fish exposed to chronic hypoxia. Here, we saw no change in the mRNA expression of two CPT-1 isoforms ($CPT_{Til}-1$ *iso1*, $CPT_{Til}-1$ *iso2*) and a transient increase of $PPAR_{Til}\alpha$ in heart of tilapia exposed to hypoxia for 24 h (Table 4), so transcriptional downregulation of genes for enzymes involved in fatty acid oxidation may occur only after long-term acclimation to hypoxia. Similarly, mammalian studies show that mRNA expression of fatty acid oxidation genes (including *CPT-1* and *PPARα*) in heart decreases after chronic but not acute in vivo hypoxia exposure (17). The transient increase of $PPAR_{Til}\alpha$ (Table 4) was remarkably similar to that seen for $PDK_{Til}-2$, suggesting related transcriptional control of these genes as seen in mouse hypoxic skeletal muscle (2).

Depression of fatty acid oxidation by other means during hypoxia exposure in tilapia is potentially important in contributing to metabolic rate depression and avoiding the potential for harmful effects of lipotoxicity and uncoupling of glucose oxidation associated with uncontrolled fatty acid oxidation in hypoxic and reperfused mammalian myocardium (13, 16). In particular, the large and rapid decrease of plasma [NEFA] (Fig. 5*C*) strongly implicates substrate supply as a major regulator of fatty acid oxidation in heart and other tissues of tilapia during hypoxia exposure. NEFA are a metabolically dynamic lipid fraction of the blood and are important for fatty acid oxidation (24). A ~80% decrease in NEFA supply to tissues, as seen in this study, could therefore significantly reduce substrate oxidation and $\dot{M}O_2$ of tissues and, as also suggested by Magnoni et al. (32), may contribute to metabolic rate depression. It may also lessen potential effects of impaired fatty acid oxidation in hypoxia. Whatever the significance, several hypoxia-tolerant fishes including the Mozambique tilapia show a decrease in plasma [NEFA] during oxygen deprivation (56, 57, 60) that appears to be due to a norepinephrine-mediated inhibition of lipolysis (60). Despite reductions in plasma [NEFA], our measurements of carnitine esters in the heart provide no evidence of decreased fatty acid oxidation, but metabolite concentrations are limited in their ability to explain flux (23), so further studies on fatty acid oxidation in hypoxic isolated hearts are warranted. Whether other cellular mechanisms, such as fatty acid import or inhibition of CPT-1 via malonyl CoA, are important in limiting fatty acid oxidation is unknown and should be investigated.

Table 5. *AMPK activity in heart of tilapia exposed to normoxia or ≤8-h severe hypoxia (5% air saturation)*

	Normoxia	Hypoxia		
		1 h	2 h	8 h
AMPK	97.5±38.6[a]	58.4±16.9[a]	181.9±46.7[a]	63.8±23.8[a]

Data (in pmol·min^{-1}·mg protein^{-1}) are means ± SE (n = 3–5). AMPK, AMP-activated protein kinase. Values with different superscripted letters are significantly different ($P < 0.05$, 1-way ANOVA with Holm-Sidak test).

Activation of AMPK has recently been implicated in reducing ATP demand of certain tissues as well as whole animal metabolic rate depression in hypoxia-exposed goldfish and crucian carp (28, 52). Our results do not suggest a role for AMPK in hypoxia tolerance of tilapia heart (Table 5), and the absence of an increase in [AMP_{free}] (Table 3) is consistent with the lack of AMPK activation. AMPK also was not hypoxia responsive in goldfish hearts (28). Increases in cardiac AMPK activity may not be necessary in hypoxic fishes because ATP demand can be greatly reduced via decreases in Q, as observed in this study (Fig. 2, *C* and *D*). AMPK also may be more important for anoxia tolerance than for hypoxia tolerance (52).

Perspectives and Significance

Hypoxic cardiac failure due to a mismatch between ATP supply and demand can quickly result in the death of hypoxia-sensitive animals, including most endotherms. Hypoxia-tolerant species, however, possess the ability to balance cardiac ATP supply with demand and downregulate cardiac ATP turnover to ensure survival during hypoxia exposure (20). In the heart of the hypoxia-tolerant tilapia, our measurements indicate that an integral component of hypoxia-induced metabolic rate depression is a rapid, sustained depression of CPO (i.e., cardiac ATP demand) achieved via a hypoxia-responsive bradycardia. The depression of CPO brings the ATP demand of the tilapia heart below the estimated level sustainable by anaerobic glycolysis alone, in agreement with the schema of Farrell and Stecyk (20). These findings support the argument

that hypoxic bradycardia in fishes serves an important, potentially adaptive, role in hypoxia tolerance of the heart, as well as of the whole organism (19).

Responses of cardiac ATP supply pathways during hypoxia exposure were usually slower and less well defined. These results suggest that depression of ATP demand, rather than ATP supply, is more important in controlling the downregulation of ATP turnover in the heart of hypoxia-tolerant fishes exposed to low oxygen. Modulation of ATP supply may be important for other aspects of hypoxia tolerance, however. The PDH/PDK pathway, for example, may be important for mitigation of ROS damage at least during acute exposure (2, 12). Finally, the marked reduction of circulating [NEFA] observed stands out as an ATP supply pathway that should be further investigated to ascertain its potentially major importance in contributing to metabolic rate depression and preventing lipotoxicity in tissues during hypoxia.

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DISCLOSURES

No conflicts of interest are declared by the author(s).

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