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Roles of nitric oxide, nitrite and myoglobin on myocardial efficiency in trout (*Oncorhynchus mykiss*) and goldfish (*Carassius auratus*): implications for hypoxia tolerance

Claus Lunde Pedersen, Serena Faggiano*, Signe Helbo, Hans Gesser and Angela Fago†

Department of Biological Sciences, Building 1131, Universitetsparken, Aarhus University, DK-8000, Aarhus C, Denmark

*Present adress: Dipartimento di Biochimica e Biologia Molecolare, Università degli Studi di Parma, 43100 Parma, Italy

†Author for correspondence (angela.fago@biology.au.dk)

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SUMMARY

The roles of nitric oxide synthase activity (NOS), nitrite and myoglobin (Mb) in the regulation of myocardial function during hypoxia were examined in trout and goldfish, a hypoxia-intolerant and hypoxia-tolerant species, respectively. We measured the effect of NOS inhibition, adrenaline and nitrite on the O_2 consumption rate and isometric twitch force development in electrically paced ventricular preparations during hypoxia, and measured O_2 affinity and nitrite reductase activity of the purified heart Mbs of both species. Upon hypoxia (9% O_2), O_2 consumption and developed force decreased in both trout and goldfish myocardium, with trout showing a significant increase in the O_2 utilization efficiency, i.e. the ratio of twitch force to O_2 consumption, suggesting an increased anaerobic metabolism. NOS inhibition enhanced myocardial O_2 consumption and decreased efficiency, indicating that mitochondrial respiration is under a tone of NOS-produced NO. When trout myocardial twitch force and O_2 consumption are enhanced by adrenaline, this NO tone disappears. Consistent with its conversion to NO, nitrite reduced O_2 consumption and increased myocardial efficiency in trout but not in goldfish. Such a difference correlates with the lower O_2 affinity measured for trout Mb that would increase the fraction of deoxygenated heme available to catalyze the reduction of nitrite to NO. Whereas low-affinity trout Mb would favor O_2 diffusion within cardiomyocytes at high *in vivo* O_2 tensions, goldfish Mb having higher O_2 affinity and higher nitrite reductase activity appears better suited to facilitate O_2 diffusion and nitrite reduction in the heart during severe hypoxia, a condition particularly well tolerated by this species.

Key words: heart, contractile force, oxygen consumption, hypoxia.

INTRODUCTION

Because of fluctuations in water O_2 levels and of different lifestyles, fish show probably the widest range of adaptive responses to changes in either ambient or tissue O_2 availability among vertebrates. In particular, crucian carp (*Carassius carassius*) and goldfish (*Carassius auratus*) have evolved to tolerate prolonged and severe hypoxia when overwintering in ice-covered ponds and are recognized, together with freshwater turtles, as probably the most hypoxia-tolerant vertebrates (Bickler and Buck, 2007). Other species, including trout (*Oncorhynchus mykiss*), have evolved as fast swimmers that rely on a steady tissue O_2 supply and are unable to tolerate even short hypoxic episodes. Like all vertebrates, fish must retain cardiac performance under highly variable ambient and physiological conditions and thus represent well-suited models to study how cardiac function is maintained when O_2 availability becomes a limiting factor.

As in other tissues, the O_2 tension in cardiomyocytes is determined by the balance between the supply of O_2 from the blood and the O_2 consumption by the mitochondria. The O_2 carrier myoglobin (Mb), typically expressed at high levels in cardiac and skeletal muscles, facilitates the diffusion of O_2 from the extracellular space to the mitochondria (Wittenberg and Wittenberg, 2003), particularly when extracellular O_2 tensions are low, as shown in the fish heart (Canty and Driedzic, 1987).

Among the factors regulating O_2 consumption, nitric oxide (NO) appears to be of crucial importance in regulating the rate of respiration during hypoxia. NO is a signaling molecule with a widespread mode of action, playing an important physiological role in limiting mitochondrial O_2 consumption in tissues (Erusalimsky and Moncada, 2007). In heart muscle this effect has been shown to be associated with an increased O_2 utilization capacity, in terms of the mechanical activity per O_2 consumed (Shen et al., 2001; Misfeldt et al., 2009). Furthermore, the effects of NO on myocardial O_2 consumption appear to be prominent under hypoxia (Misfeldt et al., 2009) and may relate to the fact that NO binds competitively with O_2 to cytochrome *c* oxidase (Cleeter et al., 1994; Brown and Cooper, 1994; Erusalimsky and Moncada, 2007). As NO limits O_2 consumption, it is of interest to examine how it acts in combination with factors involved in the upregulation of the cellular energy demand. For some fish species like trout, hypoxia may often be associated with adrenergic stimulation of heart muscle contractility and a previous study suggests that the response to such a stimulation during hypoxia is much stronger in rainbow trout than in the endothermic rat (Nielsen and Gesser, 2001).

Although hypoxia favors the reduction of mitochondrial respiration *via* NO, it may also limit NO generation from the nitric oxide synthase (NOS) reaction, where O_2 is a substrate together with L-arginine (L-Arg). Under such conditions NO may originate from nitrite, which is a natural product of the metabolism of NO and can be taken up from food or water by the gills of freshwater fish (Jensen, 2009). Nitrite can be converted back to NO by the catalytic activity of numerous metal-containing enzymes, including Mb that in the deoxy form may function as a nitrite reductase (Shiva et al., 2007). Due to its abundance in the heart, Mb has an important

role in the generation of NO from nitrite during hypoxia in the mammalian heart, where it may prolong O_2 availability and thereby have a protective effect against heart ischemia (Rassaf et al., 2007; Hendgen-Cotta et al., 2008; Cossins and Berenbrink, 2008). Evidence for NOS activity and nitrite conversion to NO in the fish heart has been provided in recent studies, where the effects of NO in the modulation of cardiac function have been studied (Garofalo et al., 2009; Tota et al., 2005; Imbrogno et al., 2001; Cerra et al., 2009). However, the functional effects of NO generated by the NOS reaction or from nitrite have not been investigated so far in the fish heart muscle subjected to hypoxia.

In this study, we examined the effects of NOS inhibition or nitrite addition on O_2 consumption, twitch force and O_2 utilization efficiency in hypoxic ventricular ring preparations isolated from goldfish and rainbow trout, which are tolerant and intolerant to hypoxia, respectively. Considering the difference in hypoxia tolerance between rainbow trout and goldfish, we first investigated the effects of hypoxia on O_2 consumption and mechanical performance in cardiac ventricular muscle and how these are influenced by NO. Tissue NO is supplied by the NOS reaction as well as by the reduction of nitrite, which in the heart is primarily dependent on the degree of Mb deoxygenation and ultimately on the Mb O_2 affinity. The importance of both of these NO sources in the heart muscle was examined by applying either asymmetric dimethylarginine (ADMA), which inhibits NOS, or nitrite in the presence of ADMA (to inhibit concurrent NOS-catalyzed NO production). Furthermore, in view of the strong effect of adrenaline on trout heart muscle contractility during hypoxia, we examined the effects of NO on O_2 consumption and mechanical performance in the presence of adrenaline. Finally, we measured O_2 affinity and nitrite reductase activity of the Mb purified from the heart muscle of the two species and correlated these results with those obtained with the ventricular preparations to elucidate the functional role of Mb as an O_2 carrier and nitrite reductase in the hypoxic fish heart.

MATERIALS AND METHODS

Experimental animals and myocardial preparations

Trout (*Oncorhynchus mykiss* Walbaum) and goldfish (*Carassius auratus* Linnaeus) were kept in freshwater tanks at 16–18°C and fed regularly. On the day of the experiment, the animals were captured and decapitated, and the heart was quickly transferred to an ice-cold physiological solution, where a ring-shaped preparation of ~10 mm in diameter was prepared from the ventricle. The physiological solution consisted of (mmol l^{-1}): NaCl (125), KCl (2.5), $MgSO_4$ (0.94), $NaHCO_3$ (15), NaH_2PO_4 (1), $CaCl_2$ (1.2) and glucose (5). Under normoxia, the solution was equilibrated with a gas mixture consisting of 49% O_2, 1% CO_2, 50% N_2, which was changed to 9% O_2, 1% CO_2, 90% N_2 to attain hypoxia. At the experimental temperature of 15°C the pH of the solution was 7.7.

Measurements of twitch force and O_2 consumption

The experimental setup has been described in detail previously (Kalinin and Gesser, 2002; Overgaard and Gesser, 2004; Misfeldt et al., 2009). Briefly, a single ring-shaped ventricle preparation was mounted on two hooks, of which one was connected to a force transducer (Fort 10; World Precision Instruments, Sarasota, FL, USA). The heart ring was placed in physiological solution in a stirred measuring chamber maintained at 15°C (close to the physiological temperature for both species) and equipped with an O_2 electrode (Radiometer E5046, Copenhagen, Denmark). The preparation was stretched to produce maximal twitch force by a micrometer screw. Twitch force was elicited by two silver electrodes placed on opposite sides of the myocardial ring and connected to a stimulator (Grass SD9, Quincy, MA, USA) providing square pulses at a rate of 0.3 s^{-1} and with a polarity that was altered between stimulations. Each stimulation had a duration of 5 ms and a voltage 1.5 times higher than that necessary to elicit full twitch force (Kalinin and Gesser, 2002).

The physiological solution was recirculated between the measuring chamber (2.56 ml) and the reservoir (20 ml), where it was continuously bubbled with a gas mixture delivered by a precision gas mixing pump (Wöstoff, Bochum, Germany). During simultaneous measurements of O_2 consumption and twitch force, recirculation of the solution was stopped and the decrease in O_2 tension in the closed chamber was recorded over time. Values of O_2 tension and force were sampled at a rate of 100 s^{-1} using a Biopac MP100 data acquisition system and analyzed using the program Acqknowledge 3.7.0 (Biopac Systems, Goleta, CA, USA). The rate of O_2 consumption was obtained by linear regression and the twitch force by the difference between the minimal (resting tension) and the maximal force developed. Calibration of the O_2 electrode was performed as described (Misfeldt et al., 2009).

The preparation was stimulated to twitch force development and maintained at 49% O_2, 1% CO_2, 50% N_2 for at least 40 min before recirculation was stopped (i.e. the measuring chamber was closed) for 20 min to measure O_2 consumption rate together with twitch force, while the O_2 content of the gas perfusing the reservoir solution was switched from 49% to 9% O_2. Then recirculation with hypoxic solution was started (i.e. the measuring chamber was again connected with the reservoir) and maintained for 20 min before it again was stopped for 20 min to measure O_2 consumption rate and twitch force. This procedure, with recirculation open and closed each for 20 min, was used to obtain O_2 consumption and twitch force during control conditions and when the preparation was exposed to different treatments, as shown schematically in Figs 1–3. The first of these treatments included (1) 150 μmol l^{-1} ADMA, an inhibitor of NOS, (2) 10 μmol l^{-1} adrenaline either in the presence or in the absence of ADMA, and (3) sodium nitrite in a concentration of 13 μmol l^{-1} (trout and goldfish) or 100 μmol l^{-1} (goldfish) in the presence of ADMA. In these experiments, the time course for the experimental (i.e. treated) and control preparations was the same. When analyzing the effect of different treatments, the values for O_2 consumption rate and developed twitch force were normalized to the corresponding values recorded in the preceding recording period (i.e. when experimental and control preparations compared were exposed to identical conditions, see Figs 1–3 for details).

The effects of the different treatments were analyzed using either paired or unpaired Student's *t*-tests as appropriate with the significance limit set at P=0.05. All chemicals were from Sigma-Aldrich (St Louis, MO, USA) and were added to the recirculating physiological solution.

Purification of heart Mb

Hearts from trout and goldfish were cut into small pieces that were washed three times free from blood at low speed centrifugation in 0.9% NaCl and homogenized on ice in 50 mmol l^{-1} Tris buffer, 0.5 mmol l^{-1} EDTA, pH 8.0. The homogenate was centrifuged and solid ammonium sulphate was slowly added to the supernatant to reach 60% saturation. After stirring for 30 min at 5°C, the suspension was centrifuged and the protein in the supernatant was again precipitated with 80% ammonium sulphate. After centrifugation, the pellet was resuspended in 5 mmol l^{-1} Tris buffer, 0.5 mmol l^{-1} EDTA, pH 8.5 and dialyzed against the same buffer. The protein was then loaded on a pre-packed Superdex 75 column (Amersham

Biosciences, Uppsala, Sweden) and eluted with 5 mmol l^{-1} Tris buffer, 0.5 mmol l^{-1} EDTA, 0.1 mol l^{-1} NaCl, pH 8.5, where Mb separated from contaminating Hb. Eluate was monitored at 412 nm. Purity was checked by 10–15% SDS-PAGE using a PhastSystem (Amersham Biosciences). Finally, the Mb fraction was desalted, concentrated by ultrafiltration and stored in aliquots at –80°C.

O_2 equilibrium measurements

O_2 equilibrium curves of purified trout and goldfish Mbs and from horse heart Mb (Sigma-Aldrich) were determined in duplicate at 25°C using a thermostatted thin-layer gas diffusion chamber connected to precision gas mixing pumps (Wöstoff) for mixing air, pure N_2 and pure O_2 to establish stepwise changes in O_2 tension (P_{O2}) within the chamber (Weber, 1992). Samples (3–4 µl) were in 100 mmol l^{-1} phosphate buffer, 0.5 mm EDTA, pH 7.2. Absorbance was monitored at 436 nm. Before measuring O_2 equilibria, ferric horse Mb was reduced to the ferrous form with solid dithionite at 0–4°C and rapidly passed on a desalting PD10 column (Amersham Biosciences). P_{50} and n_{50} values (O_2 tensions and Hill's cooperativity coefficients at 50% saturation, respectively) were calculated from Hill plots, $\log[Y/(1–Y)]$ *vs* $\log P_{O_2}$, where Y is the fractional O_2 saturation.

Measurements of nitrite reductase activity

The reaction with nitrite was carried out in anaerobic 1 cm cuvettes sealed with a rubber cap for N_2 equilibration in the presence of sodium dithionite essentially as described by Salhany (Salhany, 2008). The presence of low levels of dithionite in the reaction mixture eliminated residual contaminating O_2 and provided the advantage of converting (ferric) metMb to the (ferrous) deoxy form without reacting with nitrite at appreciable rates, as previously shown (Salhany, 2008). The reaction of deoxygenated Mb (deoxyMb) with nitrite proceeds in steps according to the scheme:

$$\text{deoxyMb} + NO_2^- + H^+ \rightarrow \text{metMb} + NO + OH^- . \quad (1)$$

In the presence of dithionite, the metMb formed is reduced to deoxyMb:

$$\text{metMb} + \text{dithionite} \rightarrow \text{deoxyMb} . \quad (2)$$

And in a closed anaerobic cuvette, the NO generated binds to the deoxy heme:

$$\text{deoxyMb} + NO \rightarrow \text{Mb–NO} . \quad (3)$$

The protein (final concentration, 10 µmol l^{-1}) in the cuvette was in deoxygenated 100 mmol l^{-1} sodium phosphate, 0.5 mmol l^{-1} EDTA, pH 7.2, and contained 100 µmol l^{-1} sodium dithionite. Buffer and nitrite solutions were deoxygenated by bubbling with pure N_2 and a positive N_2 pressure was applied on the gas phase of the cuvette to prevent air influx. A deoxygenated solution of sodium nitrite was quickly injected in the cuvette using a gas-tight syringe and the measurement of the reaction kinetics was started immediately. To allow for comparison with other Mbs, kinetics were studied at 25°C under pseudo-first-order conditions with a 10–500-fold excess of nitrite, similar to previous studies (e.g. Shiva et al., 2007). UV-visible spectra (400–700 nm) were recorded using a diode-array HP 8543 spectrophotometer (Agilent Technologies, Karlsruhe, Germany) at different time intervals to verify that the heme changed from fully deoxygenated to fully NO during the reaction and that isosbestic points were maintained. The relative decay in the absorbance at 431 nm (the absorbance peak of deoxyMb) measured over time was fitted according to a monoexponential function to calculate the observed rate of Mb–NO formation for each nitrite concentration. Because dithionite reduces met (ferric) Mb formed in the reaction to the deoxy (ferrous) form, Mb–NO is the only product formed in the reaction of deoxyMb with nitrite, whereby fitting the change in absorbance over time yields directly the observed rate, without the need of prior spectral deconvolution (Shiva et al., 2007).

Autoxidation rates of horse, trout and goldfish Mbs

Rates of spontaneous autoxidation from oxy (ferrous) to met (ferric) Mb were measured in 100 mmol l^{-1} phosphate buffer, 0.5 mm EDTA, pH 7.2 at 37°C in air. The absorbance at 407 nm was monitored over time to measure the rate of metMb formation.

RESULTS

At full oxygenation (49% O_2), goldfish heart muscle developed similar twitch force as trout heart muscle but at a lower rate of O_2 consumption (Table 1), thereby showing a greater O_2 utilization efficiency (i.e. ratio of twitch force to O_2 consumption). Hypoxia obtained by lowering O_2 in the gas mixture from 49% to 9% entailed a significant decrease in O_2 consumption rate and force development for both species, although these decreases in relative terms were significantly larger for trout than for goldfish heart muscle (Table 1). For trout the relative myocardial O_2 utilization efficiency increased significantly under hypoxia and became similar to that for goldfish.

The effect of NOS-catalyzed NO production on O_2 consumption and force generation was studied under hypoxia (9% O_2) by adding ADMA, an inhibitor of NOS activity. In the presence of ADMA, both trout and goldfish myocardial tissues displayed a significant increase in the rate of O_2 consumption by 17% and 26%, respectively, relative to control preparations (Fig. 1A,B) whereas force was not significantly affected (Fig. 1C,D). Consistently, myocardial O_2 utilization efficiency decreased by 13% and 36% in trout and goldfish, respectively, when NOS activity was inhibited (Fig. 1E,F). These ADMA effects did not differ significantly between trout and goldfish.

Table 1. Rate of O_2 consumption, twitch force development and their ratio (O_2 utilization efficiency) in trout and goldfish myocardial preparations at 49% O_2 and 9% O_2, 15°C

	49% O_2			9% O_2			49% *vs* 9% O_2		
	O_2 consumption (µmol min^{-1} g^{-1})	Force (mN mm^{-2})	Force/O_2 consumption	O_2 consumption (µmol min^{-1} g^{-1})	Force (mN mm^{-2})	Force/O_2 consumption	ΔO_2 consumption (%)	ΔForce (%)	ΔForce/O_2 consumption (%)
Trout (*N*=28)	0.19±0.01	3.19±0.22	18.15±1.76	0.06±0.00*	1.32±0.10*	22.67±2.09	–68±2*	–60±3*	30±9*
Goldfish (*N*=16)	0.12±0.02	2.64±0.34	26.18±3.08	0.07±0.01*	1.43±0.23*	25.08±3.74	–36±8*	–42±5*	13±20
	†		†				†	†	

Data are means ± s.e.m.
*Significant difference between 49% and 9% O_2 (paired *t*-test).
†Significant difference between trout and goldfish (unpaired *t*-test).

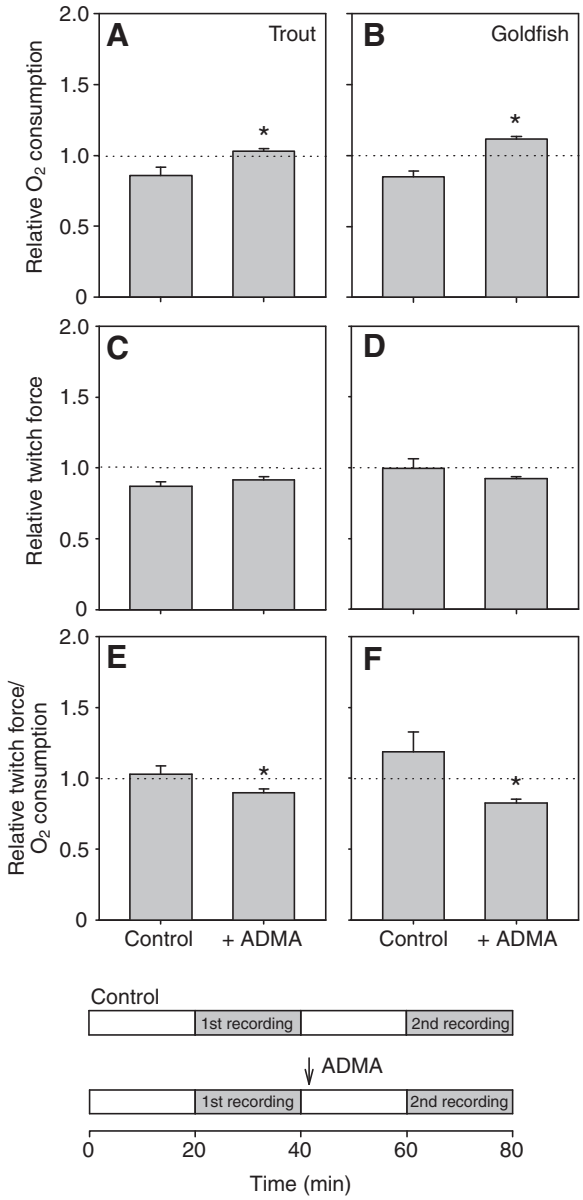

Fig. 1. O_2 consumption rate (A,B), twitch force development (C,D) and ratio of twitch force to O_2 consumption (E,F) at 9% O_2, 15°C in myocardial preparations from trout and goldfish in the presence and absence (controls) of asymmetric dimethylarginine (ADMA) (150 μmol l^{-1}). The values reported for the experimental (trout *N*=5, goldfish *N*=5) and control preparations (trout *N*=5, goldfish *N*=5) obtained during the 2nd recording period are normalized to those obtained during the 1st recording, i.e. before ADMA was applied (see horizontal bars for recording sequence). Data are means ± s.e.m. Significant differences between experimental and control preparations (unpaired *t*-test) are indicated (*P<0.05). The dotted lines show the normalized values (i.e. 1) obtained during the 1st recording.

The possibility that exogenous L-Arg, which is a substrate for NOS, could increase the effects of NO was examined in trout ventricular muscle. Addition of 100 μmol l^{-1} L-Arg did not affect O_2 consumption, twitch force or their ratio significantly (data not shown), indicating that NOS activity is not limited by L-Arg availability.

As adrenaline increases twitch force development in the anoxic heart muscle (Nielsen and Gesser, 1983), we examined the effect of NOS inhibition under hypoxia in trout cardiac muscle exposed to 10 μmol l^{-1} adrenaline, a concentration that provides near maximal stimulation of twitch force development (Nielsen and Gesser, 2001). Both twitch force development and O_2 consumption rate were significantly enhanced upon addition of adrenaline whereas O_2 utilization efficiency remained unaffected (Fig. 2). The addition of ADMA together with adrenaline had no further effect on either O_2 consumption, twitch force or their ratio.

In the hypoxic heart, NO may originate from reduction of nitrite catalyzed by deoxyMb. In order to estimate the production of NO from nitrite, we examined the effect of externally added sodium nitrite in the presence of ADMA to block NOS activity. In trout myocardial tissue, the addition of 13 μmol l^{-1} nitrite decreased O_2 consumption significantly (Fig. 3A). Nitrite did not affect twitch force (Fig. 3C) but it increased the twitch force to O_2 consumption ratio significantly (Fig. 3E). In goldfish, the addition of either 13 μmol l^{-1} or 100 μmol l^{-1} nitrite had no significant effect on O_2 consumption (Fig. 3B) or force development (Fig. 3D). However, consistent with the results on trout, the ratio of twitch force to O_2 consumption tended to increase in the presence of nitrite, although not significantly (Fig. 3F).

To investigate the role played by Mb in the changes in myocardial function induced by nitrite, we measured O_2 affinity and nitrite reductase activity of purified heart Mb from trout and goldfish. These functional properties were moreover compared with those of horse heart Mb to evidence possible differences between fish and mammalian Mbs. To allow direct comparison of the functional properties measured here with those of other Mbs published in the literature, all functional experiments were made at 25°C. As indicated in Fig. 4, O_2 equilibrium experiments showed P_{50} values at 25°C of 3.4 Torr (1 Torr≈133 Pa) for trout Mb, 1.3 Torr for goldfish Mb and 0.9 Torr for horse Mb. Assuming a constant enthalpy of oxygenation for Mb of approximately –14 kcal mol^{-1} O_2 (Antonini and Brunori, 1971), the estimated P_{50} values at 15°C (the temperature where the myocardial function was studied) are 1.5 Torr and 0.6 Torr for trout and goldfish Mb, respectively. Cooperativity of O_2 binding, as established from the slope of the Hill plot, was absent (n_{50}~1), confirming the monomeric structure of fish Mbs.

Nitrite reductase activities of purified trout and goldfish Mbs as well as of horse Mb were studied under anaerobic conditions in the presence of dithionite at different nitrite concentrations (Fig. 5). Analysis of absorbance spectra showed that Mb–NO (characterized by absorbance peaks of the Soret band at 421 nm for horse Mb and 417 nm for trout and goldfish Mbs) was generated in the reaction of deoxyMb (with an absorbance peak at 431 nm) with nitrite (not shown), confirming that nitrite was effectively reduced to NO by deoxyMb. As absorbance at 431 nm and the [deoxyMb] derived by spectral deconvolution (Shiva et al., 2007) followed the same time course, reaction rates were calculated by monoexponential fitting of the absorbance traces at 431 nm (Fig. 5A). Observed rates were plotted as a function of nitrite concentration to calculate the apparent second-order rate constant for the reaction of deoxyMbs with nitrite (Fig. 5B). Our data show that goldfish deoxyMb reacts with nitrite at a higher rate (17.4±0.6 mol^{-1} s^{-1}) than trout (5.5±0.2 mol^{-1} s^{-1}) and horse Mbs (6.9±0.3 mol^{-1} s^{-1}) under identical conditions. To investigate whether the higher reactivity with nitrite was related to a higher tendency to heme oxidation, we examined kinetics of autoxidation in air. These experiments (not shown) indicated that Mb from trout oxidizes faster than that from goldfish (t½~0.6 h and ~1.1 h, respectively) whereas horse Mb showed the slowest rate (t½~4.3 h) and was thus much less prone to oxidation.

DISCUSSION

As confirmed by our study, a general effect of hypoxia is to depress myocardial contractility and O_2 consumption rate – an effect that was particularly pronounced in trout (Table 1). At the same time, the myocardial O_2 utilization efficiency (in terms of twitch force development relative to O_2 consumption) in trout increased during hypoxia, suggesting an enhanced contribution of anaerobic energy production. Such an enhancement typically accompanies hypoxia as indicated by recordings of lactate production in heart muscle from trout and other species (Arthur et al., 1992; Driedzic and Gesser, 1994). Compared with the trout myocardium, the goldfish myocardium showed a higher efficiency of O_2 utilization during full oxygenation mainly as a result of a lower O_2 consumption. There are a number of possible reasons for this higher O_2 utilization

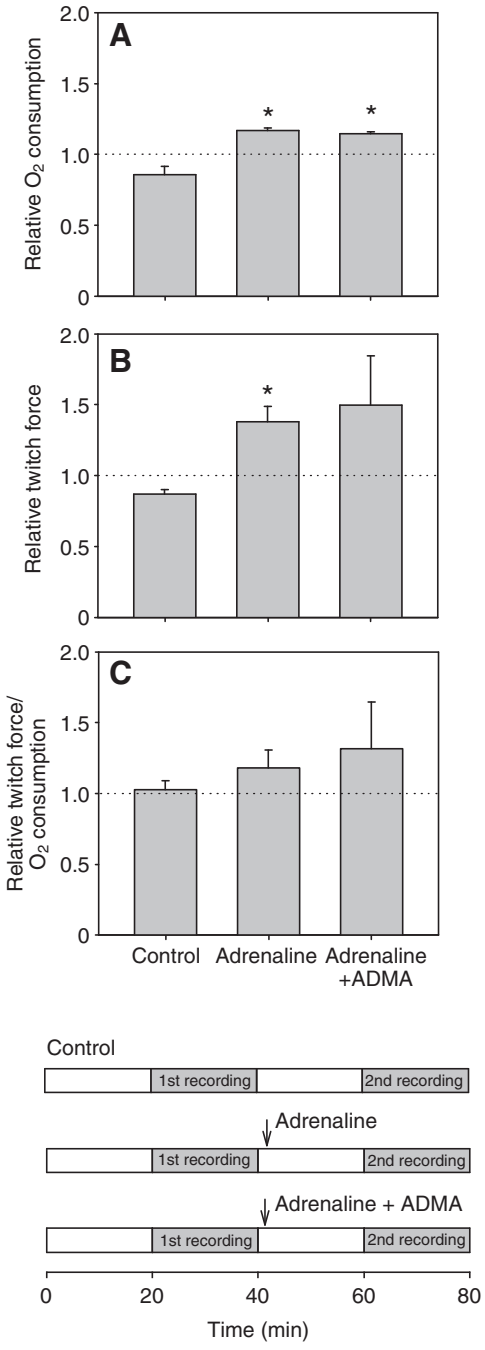

Fig. 2. O_2 consumption rate (A), twitch force development (B) and ratio of twitch force to O_2 consumption (C) at 9% O_2, 15°C in myocardial preparations from trout in the absence (control, *N*=6) and in the presence of adrenaline (10 μmol l^{-1}, *N*=5) either alone or together with asymmetric dimethylarginine (ADMA) (150 μmol l^{-1}, *N*=4). The values for the three groups obtained during the 2nd recording period are normalized to the values obtained during the 1st recording, before the applications of adrenaline and adrenaline and ADMA (see horizontal bars for recording sequence). Data are means ± s.e.m. Significant effects of adrenaline and of ADMA in the presence of adrenaline (unpaired *t*-test) are indicated (*P<0.05). There were no significant differences between the preparations exposed to adrenaline alone and adrenaline together with ADMA. The dotted lines show the normalized values (i.e. 1) obtained during the 1st recording.

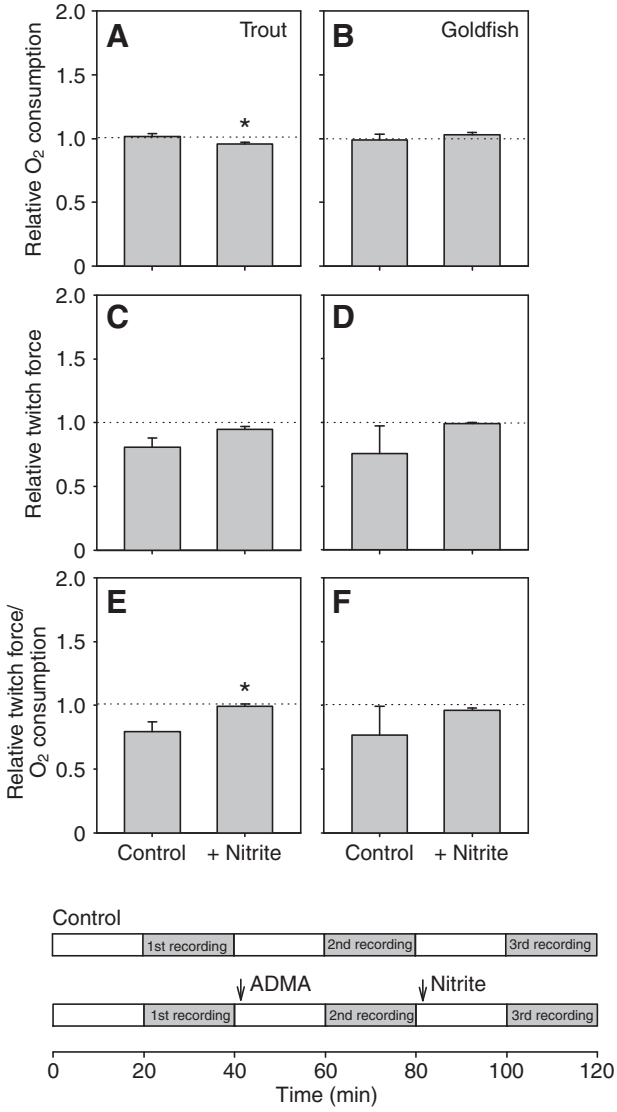

Fig. 3. O_2 consumption rate (A,B), twitch force development (C,D) and ratio of twitch force to O_2 consumption (E,F) at 9% O_2, 15°C in myocardial preparations from trout and goldfish in the absence (control: *N*=7 trout, *N*=5 goldfish) and in the presence of sodium nitrite (trout, 13 μmol l^{-1}, *N*=7; goldfish 100 μmol l^{-1}, *N*=5). All preparations were exposed to asymmetric dimethylarginine (ADMA) (150 μmol l^{-1}). The values obtained during 3rd recording are normalized to the values obtained during the 2nd recording in the presence of ADMA, before the application of nitrite (see horizontal bars for recording sequence). Data are means ± s.e.m. Significant differences (unpaired *t*-test) between ADMA+nitrite and ADMA are indicated (*P<0.05). The dotted lines show the normalized values (i.e. 1) obtained during the 1st recording.

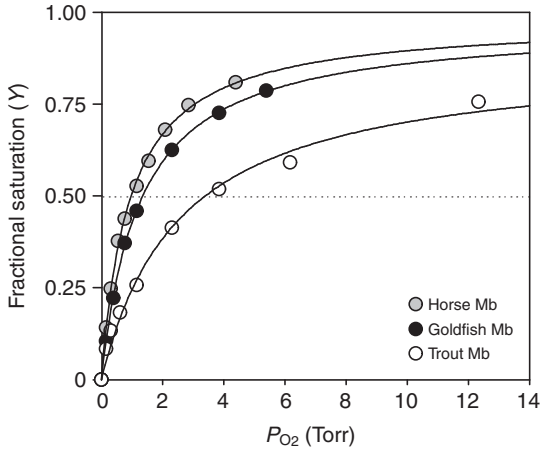

Fig. 4. O_2 equilibrium curves for horse (gray), goldfish (black) and trout myoglobin (Mb) (white) purified from heart measured in 100 mmol l^{-1} phosphate buffer, 0.5 mm EDTA, pH 7.2 at 25°C. Fitting of data according to a hyperbolic function is indicated to show non-cooperative binding. The dotted line indicates the half saturation level.

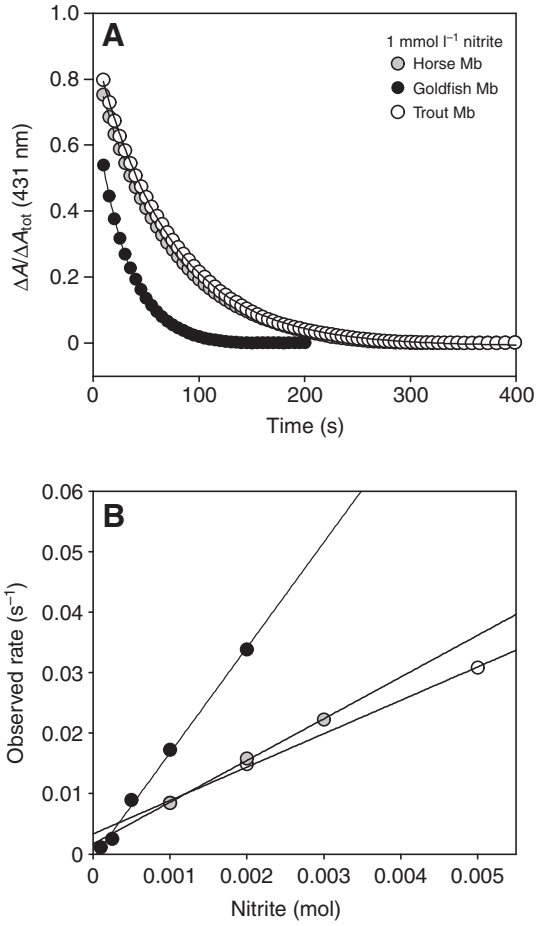

Fig. 5. (A) Representative kinetic traces of the reaction of purified ferrous myoglobin (Mb) (10 μmol l^{-1}) from horse (gray), goldfish (black) and trout (white) with 1 mmol l^{-1} nitrite measured in 100 mmol l^{-1} sodium phosphate, 0.5 mmol l^{-1} EDTA, pH 7.2, 100 μmol l^{-1} sodium dithionite under anaerobic conditions at 25°C. Each data point indicates the change in absorbance at 431 nm at time t ($\Delta A=A_t-A_{end}$) relative to the total absorbance change from time zero to the end of the reaction ($\Delta A_{tot}=A_0-A_{end}$). Fitting of data according to a monoexponential function (which gives the observed rate, s^{-1}) is indicated. (B) Plot of observed rates as function of nitrite concentrations. The second-order rate constant for the nitrite reductase activity is given by the slope of the linear regression of the data.

capacity in the goldfish myocardium, including a higher reliance on glucose/glycogen relative to endogenous fatty acids as metabolic substrates, a lower resting or non-contractile metabolism, a lower energy cost of force development, or a tighter mitochondrial coupling of the rates of O_2 consumption and ATP production. This coupling has been shown to be variable due to changes in several factors, including mitochondrial proton leak (Brand, 2005). Furthermore, the goldfish myocardium displayed smaller decreases in the rate of O_2 consumption and twitch force upon exposure to hypoxia and notably no significant change in the O_2 utilization capacity, which may reflect a lower activation of anaerobic energy production. Taken together, our data show that the goldfish myocardium differs from that of trout in that it seems able to maintain a higher degree of aerobic metabolism at low O_2 tensions without increasing anaerobic energy production. The lower O_2 consumption of the fully oxygenated goldfish heart is compatible with the observation that the heart muscle of hypoxia-tolerant species tends to have a low metabolic rate (Farrell and Stecyk, 2007). Notably, goldfish is a subspecies of the crucian carp, which shows an exceptional ability to survive and preserve heart function during prolonged anoxia (Stecyk et al., 2004).

The difference in O_2 affinity of trout and goldfish Mb may contribute to the variation in myocardial O_2 consumption observed in these species. Due to its high O_2 affinity, goldfish Mb would better function to facilitate O_2 diffusion at low O_2 tensions and thus appears better suited to maintain heart O_2 consumption rate during hypoxia. Conversely, the low O_2 affinity of trout Mb may favor O_2 unloading to the mitochondria during intense swimming under well-oxygenated ambient conditions as well as facilitated O_2 diffusion at higher *in vivo* O_2 tensions than in goldfish, but not during hypoxia. Interestingly, the value of P_{50} here reported for trout Mb correlates well with the high P_{50} values characterizing low-affinity trout blood (Weber et al., 1976). In contrast to mammalian Mbs, with P_{50} values almost invariably close to ~1 Torr at 25°C (Antonini and Brunori, 1971), as also found here for horse Mb (Fig. 4), fish Mbs span over a wider range of O_2 affinities, thus reflecting the large variability in physiological adaptations to behavioral and ambient factors found in fish species (Marcinek et al., 2001). Previous studies have shown that Mb has a critical role in maintaining heart function during hypoxia in fish (Canty and Driedzic, 1987) and that high Mb concentrations generally correlate with high cytochrome *c* oxidase activities and increase the sustainable contractile work of the heart, indicating a primary role of Mb in the intracellular diffusion of O_2 and in energy production (Wittenberg and Wittenberg, 2003). Our data (Table 1) show similar rates of O_2 consumption in working trout and goldfish ventricles when exposed to identical low O_2 levels, which suggests that trout might compensate for the lower O_2 saturation with a higher Mb concentration. Although not directly measured in this study, the low levels of Mb normally present in fish hearts (Sidell et al., 1987) may not be compatible with a significant O_2-storage function of the Mb (e.g. as during prolonged diving in marine mammals) but could buffer rapid fluctuations in blood O_2 supply. Precise determination of the Mb concentration in the heart of fish species by spectrophotometric methods that take into account distinct spectral differences of fish Mbs and Hbs are underway.

A potentially important factor in our study modulating O_2 consumption is the NO generated enzymatically by NOS or by enzymes able to convert nitrite to NO, particularly deoxyMb (Shiva et al., 2007). Regardless of its origin, NO reversibly binds to reduced cytochrome *c* oxidase competitively with O_2, and thus has a more pronounced effect on respiration rate under hypoxic conditions (Erusalimsky and Moncada, 2007). The increase in the rate of O_2 consumption observed in both trout and goldfish myocardial tissues during hypoxia in the presence of ADMA is consistent with inhibition of NOS activity by ADMA and indicates that a basal inhibitory tone of NO on respiration was relieved. Hence, these results provide further evidence that NOS is present in fish myocardial tissue and that the NO generated targets mitochondria to decrease respiration rate. According to previous studies, an inhibitory NO tone may also be associated with an improved cardiac energetic efficiency (Shen et al., 2001; Misfeldt et al., 2009), which is compatible with the lowered myocardial O_2 utilization efficiency here observed in the presence of ADMA for both trout and goldfish.

In trout myocardium in the presence of adrenaline, however, blocking NOS activity with ADMA has no significant effect on O_2 consumption. Interestingly, adrenaline induced a strong stimulation of both twitch force and O_2 consumption, demonstrating that the limits of O_2 consumption imposed by hypoxia are not fixed but adjustable. Furthermore, the relative increases in the rate of O_2 consumption and twitch force development were similar, as also indicated by the unchanged ratio of twitch force to O_2 consumption, so there was no evidence that the adrenergic stimulation enhanced anaerobic relative to aerobic metabolism, despite the hypoxic conditions. A main action of adrenaline is to increase the Ca^{2+} transient and the resulting twitch force development as well as the rate of Ca^{2+} removal and relaxation (Bers, 2002). These effects should stimulate mitochondrial respiration directly and by enhancing ATP hydrolysis and delivery of ADP and P_i to the oxidative phosphorylation. As no significant effects of ADMA were observed in the presence of adrenaline, it is possible that the strong stimulation of twitch force and O_2 consumption rate either suppresses NO formation or more likely overrides the effects of NO.

Similarly to ADMA, nitrite only influenced O_2 consumption but not force development, which is consistent with the notion that nitrite converted to NO targets mitochondria. However, the reduction in the rate of O_2 consumption induced by nitrite was small and observed only in trout myocardial tissue. In goldfish heart muscle, nitrite had no significant effect, even when added at concentrations as high as $100\,\mu mol\,l^{-1}$. That trout and goldfish differ in how nitrite affects myocardial function may be ascribed to the different O_2 affinity of their Mbs. At the O_2 levels here studied the low-affinity Mb of trout may become more readily deoxygenated and able to reduce nitrite to NO, in contrast to the high-affinity Mb of goldfish that would remain highly saturated with O_2 at the same O_2 tension. This would explain why we did not observe any effect of nitrite in the goldfish myocardium, not even when nitrite was added at high levels.

The fact that nitrite had no effect on the myocardial function of the goldfish under the conditions of the present study does not exclude that nitrite might decrease the rate of O_2 consumption under more severe hypoxia, a condition that is well tolerated by this species. When fully deoxygenated *in vitro*, purified Mbs from trout and goldfish both possess nitrite reductase activity. Whereas in trout Mb such activity ($5.5\,mol^{-1}\,s^{-1}$) is comparable with that found in horse ($6.9\,mol^{-1}\,s^{-1}$, see Fig. 5) and in other mammalian Mbs ($6\,mol^{-1}\,s^{-1}$) (Huang et al., 2005), in goldfish Mb the nitrite reductase activity is approximately 3-fold higher ($17.4\,mol^{-1}\,s^{-1}$), which is the highest reported for a globin protein so far. Interestingly, the hemoglobin of carp, a hypoxia-tolerant species closely related to goldfish, also shows a higher nitrite reductase activity than mammalian hemoglobins even at low O_2 saturations – a feature that has been related to the relatively high O_2 affinity and R-state quaternary structure of this hemoglobin (Jensen, 2009). The intermediate heme autoxidation rate of goldfish Mb compared with horse and trout Mbs, with the lowest and highest autoxidation rates, respectively, suggests that factors other than heme redox potential control the reactivity of Mb with nitrite. In analogy with neuroglobin and cytoglobin (Petersen et al., 2008), access of nitrite to the heme pocket could also play a role in modulating the nitrite reductase activity of Mb.

Although technical limitations do not allow us to accurately measure O_2 consumption rates at O_2 tensions lower than those used here, it is conceivable that in the anoxic goldfish heart, deoxyMb may effectively function to convert nitrite to NO and thus contribute to the exceptional hypoxic (and anoxic) tolerance of this animal. By contrast, trout that may periodically experience local tissue hypoxia due to intense activity, but which are intolerant to prolonged ambient hypoxia, possess Mb with O_2 affinity and nitrite reductase activity that better fit with a modulation in the mitochondrial respiration rate taking place at higher O_2 tensions.

In the present study on trout and goldfish ventricular muscle (Fig. 1) and in our previous one on turtle ventricular muscle (Misfeldt et al., 2009), NO is found to reduce O_2 consumption without any direct effects on isometric force development. This deviates from a study on perfused whole hearts from three ectothermic vertebrates, in which NOS-derived NO affected contractility in terms of stroke volume by mechanisms that remain to be clarified (Cerra et al., 2009). Possibly, this difference may relate to the fact that the perfused heart included the atrium and was allowed to contract, while the ventricular muscle of the present and previous study (Misfeldt et al., 2009) was maintained under isometric conditions. We cannot exclude that the present study, being focused on O_2 consumption and mechanical performance at tissue/cellular level, may have missed some additional effects of NO that may be preserved in the whole heart preparation, which is closer to the *in vivo* situation.

In conclusion, our results provide evidence that NOS is present in the heart tissue of trout and goldfish and it produces NO that, at least during hypoxia, maintains the O_2 consumption under a significant basal inhibitory tone. Adrenaline enhances twitch force and O_2 consumption of trout myocardium during hypoxia and drastically reduces the impact of NOS-derived NO on the respiration rate. Nitrite appears to have little or no effect on myocardial O_2 consumption under the conditions here examined, probably because of the limited availability of reactive deoxyMb, suggesting that the degree of (de)oxygenation of heart Mb as controlled by the O_2 affinity is a major determinant of the extent of the nitrite effect observed. The differences in Mb O_2 affinity and nitrite reductase activity between trout and goldfish found in this study correlate with the variations in the physiological O_2 gradients faced by these hypoxia-intolerant and anoxia-tolerant fish species.

LIST OF SYMBOLS AND ABBREVIATIONS

ADMA	asymmetric dimethylarginine
L-Arg	L-arginine
Mb	myoglobin
Mb–NO	nitrosyl myoglobin
Met	ferric form of the myoglobin
NO	nitric oxide
NOS	nitric oxide synthase
n_{50}	Hill's cooperativity coefficient

P_{O_2}	oxygen tension
P_{50}	oxygen tension at half-saturation
$t½$	half-time of reaction

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