For: Journal of Experimental Zoology A

The effects of endogenous and exogenous catecholamines on hypoxic cardiac performance in red-bellied piranhas

Running title: Myocardial hypoxia tolerance in piranhas

William Joyce[1,*], Catherine J. A. Williams[1], Sofie Iversen[1], Per Guldhammer Henriksen[1], Mark Bayley[1], Tobias Wang[1,2]

[1]*Department of Zoophysiology, Aarhus University, 8000 Aarhus C, Denmark*

[2]*Aarhus Institute of Advanced Studies, Aarhus University, 8000 Aarhus C, Denmark*

*corresponding author: william.joyce@bios.au.dk

Abstract

Catecholamines protect the fish heart during hypoxia, however, the humoral adrenergic stress response may only be invoked *in extremis*. We investigated the hypothesis that endogenous (*e.g.* neuronal) myocardial catecholamines may also impact cardiac performance during hypoxia in a hypoxia-tolerant tropical fish, the red-bellied piranha (*Pygocentrus nattereri*). Firstly, we measured endogenous tissue catecholamines and *in vitro* catecholamine release from piranha myocardium using ultra-performance liquid chromatography. Ventricle homogenates contained detectable levels of both adrenaline (7.27 ng g^{-1}) and noradrenaline (14.48 ng g^{-1}), but only noradrenaline was released from ventricular tissue incubated in Ringer's solution. Noradrenaline released in this assay was not affected by hypoxia but was promoted by the catecholamine releasing agent tyramine. Our second series of experiments explored cardiac contractile performance *in vitro* using tyramine, exogenous noradrenaline or adrenaline, and propranolol (a β-adrenoceptor antagonist). In ventricular strip preparations, β-adrenergic blockade with propranolol had no effects on twitch force or contraction kinetics in either normoxia or hypoxia, confirming that spontaneous endogenous catecholamine release did not impact cardiac performance. However, in the absence of propranolol, tyramine mimicked the positive inotropic effect of noradrenaline (10 μM) during hypoxia, although adrenaline was capable of generating larger effects. Our results suggest that, although it is not spontaneously released, inducible endogenous noradrenaline release may have a significant β-adrenoceptor dependent impact on hypoxic performance in the fish heart.

Key words: hypoxia, contractility, adrenaline, noradrenaline, fish

Introduction

Catecholamines (*i.e.* adrenaline (A) and noradrenaline (NA)) exert a positive inotropic action on the teleost heart that confers protection to cardiac performance during hypoxia (Gesser, Andresen, Brams, & Sund-Larsen, 1982; Hanson, Obradovich, Mouniargi, & Farrell, 2006; Stecyk, Larsen, & Nilsson, 2011; Farrell and Smith, 2017). In fishes, catecholamines may be released from chromaffin cells (found in the head kidney and posterior cardinal vein in teleosts), and thereby elevate plasma catecholamine concentrations during hypoxia exposure *in vivo* (Fritsche and Nilsson, 1990; Ristori & Laurent, 1989; Perry, Fritsche, Kinkead, & Nilsson, 1991). The 'acute humoral adrenergic stress response' thus appears an integrative response, however its generality and significance has been questioned (Perry & Bernier, 1999; Perry et al., 2004). The classical identifications of the response were conducted on temperate species that typically avoid environmental hypoxia and are rarely exposed to the extreme and rapidly developing environmental hypoxia as experienced by species in tropical water bodies. By contrast, in two Amazonia teleosts (traíra, *Hoplias malabaricus*, and jeju, *Hoplerythrinus unitaeniatus*), Perry et al. (2004) only observed catecholamine release during very severe hypoxia. Most strikingly, the pacu (*Piaractus mesopotamicus*) did not exhibit a significant humoral adrenergic response during hypoxia (Perry et al., 2004).

In the present study, we present the alternative hypothesis that endogenous cardiac catecholamine sources may protect myocardial performance during hypoxia. In the mammalian heart, ischemia directly triggers the release of endogenous catecholamines (Schömig, Dart, Dietz, Mayer, & Kübler, 1984, Schömig, Fischer, Kurz, & Richardt, 1987; Lameris et al., 2000) from sympathetic neurons, or non-neuronal (e.g. Huang et al., 1996) sources (Lameris et al., 2000). Considerable levels of endogenous catecholamines have also been detected in teleost hearts (Östlund, 1954; von Euler & Fänge, 1961; Temma et al., 1989) and the teleost ventricle is innervated by sympathetic (adrenergic) nerves (Yamauchi & Burnstock, 1968; Gannon & Burnstock, 1969; Stoyek, Croll, & Smith 2015), which can influence contractility (Donald & Campbell, 1982; Temma et al., 1986). Further, 'granular vesicles' have been detected in teleost cardiomyocytes suggesting that catecholamines may also be stored in non-neuronal cells (Yamauchi & Burnstock, 1968; Santer & Cobb, 1972; Laurent, Holmgren, & Nilsson, 1983).

We chose to investigate this hypothesis in heart preparations from the red-bellied piranha (*Pygocentrus nattereri*), a particularly hypoxia-tolerant teleost (i.e. critical PO_2 1.5 – 2 kPa measured *in vivo*; W. Joyce, C. J. A. Williams, H. Malte & T. Wang, unpublished data) that is closely related to pacu, which conspicuously lacks the humoral adrenergic response to hypoxia (Perry et al., 2004). We quantified endogenous catecholamines (A and NA) in

ventricle homogenates and developed an assay to measure catecholamine release in non-working myocardium incubated in Ringer's solution during normoxia, hypoxia and following exposure to the catecholamine releasing agent tyramine (Gaffney, Morrow, & Chidsey, 1962; Holmes and Fowler, 1962). Tyramine induces non-exocytotic catecholamine release from nerve terminals due to its high affinity for neuronal storage proteins, resulting in the displacement of endogenous catecholamines (Lameris et al., 1999). To investigate the functional potential of the spontaneous catecholamine release from endogenous stores during hypoxia, ventricular muscle strip preparations were deprived of oxygen in the absence and presence of propranolol (β-adrenergic receptor blockade). To directly examine the effect of endogenous catecholamine release, hypoxic and normoxic ventricular preparations were treated with tyramine. This was compared with the effects of exogenous A and NA on hypoxic and normoxic piranha myocardium.

Materials and Methods

Experimental Animals

Captive bred red-bellied piranhas (*Pygocentrus nattereri*) of both sexes weighing 379 ± 44 g (mean ± SEM, n = 13 for myocardial catecholamine measurements, n=11 for Ringer's catecholamine release assay and ventricular strip study) were obtained from a private collection and held at Aarhus University at 27°C. The photoperiod was maintained at a 12L:12D cycle and the fish were fed commercial fish food twice a week. In all experiments, ventricles were harvested from fish euthanized with benzocaine (0.25 g L^{-1}) followed by severance of the spinal cord. The studies were performed in accordance with Danish animal care legislation.

Chemicals

PCA: perchloric acid

AMNA: alpha-methyl noradrenaline

A: adrenaline

NA: noradrenaline

EGTA: ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid

Glutathione

DPE: 1,2-diphenylethylenediamine

CAPS: 3-cyclohexylamino-1-propanesulphonic acid

Ringer's Solution

NaCl (110 mM), $NaHCO_3$ (10 mM), KCl (4 mM), $MgSO_4$ (1 mM), NaH_2PO_4 (1 mM), CaCl (2 mM), and glucose (5 mM). During oxygenated conditions (normoxia), the Ringer's solution was bubbled with 50% O_2, 49% N_2 and 1% CO_2 using a Wösthoff gas-mixing pump (Bochum, Germany) which resulted in a pH of 7.56. This gas mixture (resulting in a PO_2 of ~50 kPa) exceeds the PO_2 that the heart experiences *in vivo* but compensates for the lower oxygen capacitance of saline when compared to blood (containing the oxygen-binding protein haemoglobin). Further, it was chosen for consistency and to facilitate comparisons with analogous previous work (e.g. Joyce, Simonsen, Gesser, & Wang, 2016), although an 'optimal PO_2' for *in vitro* study of the fish heart has not yet been definitively established. In perfused trout hearts, there was no significant difference in maximum cardiac performance when perfusate was gassed with room air (20.95% O_2) or extremely hyperoxic gas mixture (95.5 % O_2) (Hanson et al., 2006), thus we believe our protocol adopts an appropriate intermediate. During hypoxia, the 50% O_2 was exchanged for N_2, which we have previously verified results in a PO_2 of <1.5 kPa in this system (Joyce, Gesser, Bayley, & Wang, 2015).

Endogenous catecholamine determinations

Endogenous Myocardial Catecholamines in Ventricular Tissue

The heart was dissected from euthanized fish and placed in 30 ml of Ringer's solution to remove excess blood including plasma catecholamines. The ventricle was then isolated, weighed, and immediately frozen in darkened Eppendorf tubes at -80°C until catecholamine determination. Frozen ventricles were homogenised at 4°C in piranha Ringer's solution at 10µL/mg tissue, with 0.2M EGTA and 0.2M Glutathione (50 µL EGTA+Glu/mL ringers) and centrifuged at 4°C for 10 mins at 9000G (TissueLyser LT, Qiagen). 200 µL of supernatant was then processed for catecholamine determination, using solid phase extraction (SPE) column, ultra-performance liquid chromatography (UPLC) separation with fluorometric detection, modified from the method of Fujino, Yoshitake, Kehr, Nohta, & Yamaguchi (2003) to derivatize catecholamines only.

Briefly, SPE columns (Supelclean™ LC-WCX SPE Tube, Sigma Aldrich) in combination with a vacuum chamber (Supelco, Visiprep, Merck) were activated with 0.5ml 0.5M HCl, and washed with 1 ml water, the sample was added and filtered through the column with 0.8ml water at a rate of 0.25ml/min. The column was double washed

in water (1ml) before the sample was eluted with 0.2M PCA containing 25nM internal standard (AMNA). 50 µL of elutant was mixed with 200 µL MilliQ water. All preparation was in darkened Eppendorf tubes in a low-light laboratory.

Ringer's samples for catecholamine release determination

Ventricles were removed from euthanized fish, and half the ventricle (~100 mg) placed in darkened Eppendorf tubes in oxygenated Ringer's solution (1.5 mL/100 mg tissue) for 30 min. Ringer's samples were taken at three sequential time points: i) after 30 min with oxygenation, ii) after 30 min in hypoxia, and iii) after 30 min in reoxygenated solution after addition of tyramine (0.3 mM). At each sampling, a 0.45 mL aliquot of Ringer's was taken, to which we added 0.05 ml internal standard (AMNA) giving 0.5mL volume containing 50nM AMNA. These samples were derivatized without SPE preparation.

Sample derivatisation and detection

1 mM A, NA and AMNA Standards were prepared in 0.1M PCA and combined stock solutions of 25nM (concentration of PCA 2.5 µM) stored at -80^{o}C until the day of use. Derivatisation agents were prepared fresh every 2 weeks. CAPS buffer contained i) 2 parts 90% vol/vol water in methanol, with ii) 6 parts 0.3M CAPS in 90% vol/vol water in methanol, pH adjusted to 10 with 1M NaOH, iii) 3 parts potassium hexacyanoferrate (III) 20 mM in 50% vol/vol methanol in water and iv) 24 parts methanol. DPE buffer was prepared from 0.1M DPE in 0.1M HCl, mixed 2:1 v/v with 0.3M glycine. Derivatisation was achieved in a (1:1 v/v) mixture of CAPS buffer and DPE buffer. Derivatization media (total volume 80 µL) was vortex mixed with the sample (40 µL), before being heated to 50^{o}C for 20 mins, immediately chilled to 4 ^{o}C and introduced to the UPLC.

Samples were run on ACQUITY UPLC (Waters) using HSS T3 UPLC column with an isocratic flow starting at 0.111 ml/min, then at 4.83 minutes increasing linearly to 0.230 ml/min at the stop time at 12 minutes. Mobile phase was 1mM octanesulfonic acid sodium salt dissolved in 15mM sodium acetate buffer pH4.5 mixed 34:66 v/v with acetonitrile. Sample temperature was recorded at 4-9^{o}C, and column temperature ambient (23-28^{o}C). Flurometric detection was at 480 nm with excitation at 345 nm. Two injections were made per sample. Each

derivatization batch was bracketed by derivatization checks of 25 nM standard (A, NA, AMNA), and the sample set from SPE, bracketed by two 25 nM standards and a blank that was processed simultaneously with the samples.

Data analysis for catecholamine determination

The area of all peaks were determined on Waters proprietary software. For tissue determination, as small baseline fluctuations occur near NA and A peaks, the detection-time for these was set using the 25 nM 200 µl SPE-standard. The total area within these limits were determined on all SPE blanks and samples. The areas of the samples were then normalized with the AMNA area in the derivatized blank, and the area of the SPE blank at NA and A detection times subtracted from the area of the samples and the SPE-standards. The concentration of NA and A was calculated using the response of the derivatization-check standard. The concentration of NA and A in 200 µL was calculated from the 25nM SPE standards, and data presented as ng/g wet ventricular tissue. Detection limits for tissue samples as calculated from 2 x std of blanks treated as samples were 3.69 ng/g for NA and 4.01 ng/g for A. A repeated-measures one-way analysis of variance (ANOVA) and a Tukey's multiple comparisons post-hoc test were used to compare NA release during oxygenated conditions, hypoxia, and after tyramine treatment.

In vitro myocardial performance

The ventricle was excised and four strips were prepared from each fish. Each strip was cut in a longitudinal direction and tied at each end with 4/0 surgical silk. The preparations were then secured between a metal rod connected to a force transducer (Fort 10, World Precision Instruments, Sarasota, FL) and one of two silver electrodes. The signals were digitized using a Biopac MP100 (Biopac Systems, Goleta, CA, USA) data-acquisition system and sampled at 100 Hz using AcqKnowledge software. When secured, the preparations were lowered into water-jacketed organ baths containing 50 mL oxygenated Ringer's solution. The temperature was held at 27°C during the experiment. The two electrodes were connected to Grass SD9 stimulators (Quincy, MA) providing 5 ms pulses at double the threshold voltage required to elicit contraction. Stimulation frequency was initially set to 0.5 Hz, in accordance with similar previous studies on tropical fishes (Bailey, Val, Almedia-Val, & Driedzic, 1999; Iversen et al., 2013; Joyce et al., 2015). The preparations were then stretched to attain maximum force of contraction and allowed to stabilize for 20 min.

Tolerance to Hypoxia and the Effect of Endogenous Catecholamines

After the initial stabilization, propranolol (1µM) was added to half of the preparations to block the effects of released endogenous catecholamines, and all preparations left to stabilize for another 30 min. Hypoxia tolerance was investigated by replacing the O_2 in the Ringer's solution with N_2 for 30 min (*i.e.* 99% N_2 and 1% CO_2) in two of the preparations; one with propranolol (1µM) and one without (control) during each trial. The other two preparations served as time-matched controls (*i.e.* they experienced the same protocol but in the absence of hypoxia).

To evaluate the effect of stimulation frequency (*i.e.* heart rate) in *P. nattereri*, a force frequency trial was then conducted in all preparations. In each trial, the stimulation frequency was decreased to 0.2 Hz, then increased with an interval of 0.2 Hz up to a maximum of 1.4 Hz and then decreased back to 0.5 Hz. The preparations were left to stabilize force at each new frequency. We ascertained that after the force-frequency trials, force recovered to baseline values (i.e. there was no significant difference ($p>0.05$) in twitch force at 0.5 Hz immediately before and after the trials in both normoxia- and hypoxia-exposed preparations). 5 min after the first force frequency trial, a saturating dose of tyramine (0.3 mM) was added to all preparations to maximally stimulate the release of endogenous catecholamines (Temma et al., 1986; 1989). 10 min after addition of tyramine, a second force frequency trial was run with the same frequencies. These *in vivo* heart rate ranges accord with heart rates recorded in the closely related pacu (30 to 90 beats min^{-1}, i.e. 0.5 – 1.5 Hz; Leite et al., 2009). A schematic diagram endogenous catecholamine release protocol is presented in Supplementary Figure 1.

Effect of exogenous catecholamines

In this protocol, stabilization of ventricular strips and hypoxia treatment was identical to that described above for the investigation of endogenous catecholamine release, although no propranolol was added. Two normoxic and two hypoxic preparations were run in parallel, before one of each was exposed to either exogenous A or NA. After hypoxia, force-frequency trials were conducted before and after A or NA dose-response curves (i.e. 10 nM, 100 nM, 1 µM and 10 µM). After each addition of catecholamine, the preparations were left for two minutes for force to stabilise before the next dosage. During the addition of the catecholamines, the preparations were stimulated at 0.5 Hz. A schematic diagram exogenous catecholamine exposure protocol is presented in Supplementary Figure 2.

Data Analysis for Myocardial Strip Preparations

The length and weight of the strips were measured at the end of each experiment to calculate the cross-sectional area of each preparation (assuming a density of 1.00 g cm^3), to which twitch force was normalized. The strips were 5.9 ± 0.2 mm in length and weighed 6.9 ± 0.6 mg (mean ± SEM). The rates of contraction and of 50% relaxation were estimated from maximum twitch force, the time to peak force and the time to 50% relaxation, respectively.

Significant differences in initial (30 min pre-hypoxia) absolute force, rate of contraction, rate of 50% relaxation, and cross-sectional areas between the treatment groups were examined using one-way ANOVAs followed by Tukey's multiple comparisons post-hoc tests. None of the values differed significantly from each other in either of the experimental protocols (Tables 1 and 2). Therefore, the results are presented as relative (%) changes from the start of the experiment (i.e. 30 min pre-hypoxia, immediately before propranolol was added in some trials; see triangular symbols in Supp Fig. 1 and Supp. Fig. 2).

Three-way ANOVAs were used to make statistical comparisons when three independent factors were simultaneous changed. A three-way ANOVA was used to investigate differences between normoxia/hypoxia, pharmacological treatments (tyramine), and different time points during hypoxia (every 5 minutes from start to end of experimental protocol). Three-way ANOVAs were also used to investigate differences between normoxia/hypoxia, pharmacological treatment and either different frequencies during force-frequency trials, or different dosages during dose-response trials. All statistical analysis was performed in GraphPad Prism (Version 7.0a). Differences were considered significant when $P < 0.05$. All values are presented as means ± SEM.

Results

Endogenous Myocardial Catecholamines

NA was the predominant endogenous catecholamine and was detected in ventricular homogenates at approximately twice the concentration of A (Table 3). During incubation in Ringer's assay, only NA was detected to be released from the myocardium (Table 4). Although the mean NA release appeared to increase during hypoxia, this was largely because of one extremely high value and thus the difference was not statistically

significant (Table 4; p=0.61). However, tyramine significantly increased NA release compared to normoxic untreated preparations (Table 4; p=0.03).

Tolerance to Hypoxia and the Effect of Endogenous Catecholamines

At the end of the hypoxia exposure, twitch force had decreased to 30% of the initial force, which was approximately half of that in the time-matched control preparations (60% initial force) (Fig. 1A). Thus, there were highly significant effects of both hypoxia ($p<0.001$) and time ($p<0.001$) ~~alone, and a significant interaction between hypoxia and time ($P<0.001$)~~ on twitch force (Fig. 1A), rate of contraction (Fig. 1B) and rate of 50% relaxation (Fig. 1C). There was no significant effect of propranolol ~~or any interaction involving propranolol ($P>0.05$)~~ on twitch force (Fig. 1A), rate of contaction (Fig. 1B), or rate of 50% relaxation (Fig. 1C), suggesting that propranolol induces no clear effect on any aspect of cardiac performance during hypoxia or normoxia.

~~. There was likewise no overall effect of propranolol on the rate of contraction, but there was a significant interaction between oxygenation and propranolol ($p=0.003$), which reflects hypoxic propranolol treated preparations exhibiting a rate of contraction approximately 10 % lower than untreated hypoxic preparations (Fig. 1B). However, this difference had already manifested at the start of hypoxia exposure (Time 0 on Fig. 1B), at which point both hypoxic and normoxic preparations had identical treatment history (i.e. before pre gas-mixture change). As there was no significant effect of propranolol on the preparations destined to become normoxic, this finding should be regarded as neither a true effect of propranolol nor an interaction between propranolol and hypoxia exposure. Together these data.~~

The force-frequency trial before the addition of tyramine revealed a negative force-frequency relationship (effect of frequency: $p< 0.001$) in both hypoxia and normoxia (Fig 2A). However, in all cases, force at 1.4 Hz was at least 50% of that at 0.2 Hz. ~~There was no interaction between frequency and oxygenation ($p=0.99$), i.e. the force-frequency relationship was similar during normoxia and hypoxia.~~ Stimulation frequency had no effect ($p>0.05$) on the rate of contraction (Fig. 2C) or rate of 50% relaxation (Fig. 2E). In all cases the depressed force and contraction kinetics associated with hypoxia were maintained across frequency.

Before the next force-frequency trial, the preparations were exposed to tyramine (0.3 mM), the effect of which was significantly reduced in propranolol treated preparations (± propranolol: $p<0.002$ for twitch force, rate of contraction, rate of 50% relaxation), indicating that propranolol blocked the positive inotropic effect of tyramine

(Fig. 2B,D,F). Following tyramine treatment, non-blocked preparations generated forces approximately 10-20 % greater than propranolol-treated preparations during hypoxia, although there was little difference under normoxic conditions. ~~and there was a corresponding significant interaction between oxygen and propranolol (P<0.02). However, we do not interpret this as a greater effect of tyramine under hypoxic conditions because normoxic propranolol treated preparations generated 10% lower force than untreated preparations before tyramine (although there was no statistically significant effect of propranolol, P>0.05).~~ To further quantify this change, we also plotted the data as the change in force, rate of contraction and rate of 50% relaxation at each contraction frequency before and after tyramine (Figure 3). For both hypoxic and normoxic preparations, the change in force before and after tyramine was similar: in untreated preparations, force increased by about 5%, whereas in those treated with propranolol force fell (due to the persistent time-dependent deterioration) by about 5 % across frequencies (Fig 3A). Thus, in both hypoxia and normoxia, tyramine had a β-adrenergic receptor dependent positive inotropic effect of almost 10 %. These effects were paralleled in contraction kinetics (Fig. 3B and 3C).

Effect of Exogenous Catecholamines

In the second experiment, hypoxia had a similar depressant effect and the force-frequency trials before the addition of exogenous catecholamines were similar to those in the tyramine experiments (Fig. 4A). A and NA both elicited dose-dependent positive inotropic actions ($p<0.001$). NA had relatively small inotropic effects (10 % increase in force) that were only revealed at the highest concentration (10μM). By contrast, A maximally increased force by over 50% in normoxia. Thus, A had a significantly ($p<0.002$) greater effect than NA (Fig. 4B). During hypoxia, A increased force from approximately 25 % initial to 45 % initial, which was approximately half-way to the 65 % of initial force exhibited by time-dependent controls before the addition of catecholamines (Fig. 4B). After catecholamine treatment the negative force-frequency effect ($p<0.05$) was preserved.

Discussion

Endogenous catecholamines are present in the piranha heart and NA release is increased by tyramine

The concentration of catecholamines in the piranha heart (A: 7.27 ng g^{-1}; NA 14.48 ng g^{-1}) accord very well with carp heart (A: 3.2 ng g^{-1}; NA 16.2 ng g^{-1}; Temma et al., 1989), while a greater A concentration has been reported in cod heart (170 ng g^{-1}; von Euler and Fänge, 1961). Only a small fraction (0.3-0.9 %) of the total NA pool was

released per min in the catecholamine release assay, but the rate of release was significantly increased upon addition of tyramine. A was undetected in the Ringer's samples before or after tyramine and was thus apparently less readily mobilised from the myocardium.

Effect of catecholamines on in vitro cardiac performance

Approximately half of maximum twitch force could be provided during hypoxia, *i.e.* after 30 min of hypoxia exposure ventricular strips contracted at about 50% of the force of normoxic controls. This demonstrates that the piranha ventricle is relatively hypoxia tolerant in comparison to most other fishes (Joyce et al., 2015). The finding that tyramine, NA and A all increased twitch force suggests that, even during hypoxia, there is an additional contractile reserve that is recruitable through release of endogenous or exogenous catecholamines. This may be achieved by augmenting activator Ca^{2+} availability, as adrenergic stimulation has previously been shown to increase L-type Ca^{2+} current (Vornanen, 1998) and promote sarcoplasmic reticulum Ca^{2+} release (Cros et al., 2014) in the fish heart.

Propranolol had no direct effects on myocardial hypoxia tolerance. This, along with the absence of any significant difference in NA release between oxygenated and hypoxic treatments, indicates that catecholamines are not spontaneously released from the myocardium during hypoxia and further that if they are, they do not have a functional effect on contractility. This observation contrasts with the elevated interstitial myocardial catecholamines reported during ischemia in mammals (Schömig et al., 1984, 1987; Lameris et al., 2000), but is in accordance with the earlier finding that catecholamine secretion is unaffected by hypoxia in perfused lungfish hearts (Perry et al., 2005). However, we caution that the lungfish hearts in this previous study were only perfused with hypoxic saline for 6 min, whilst rats show no discernable catecholamine release before at least 10 min of ischemia (Schömig et al., 1984) and future studies in fishes should therefore consider the effects of more prolonged hypoxia. It is also possible that the combination of acidosis and hypoxia during ischemia triggers catecholamine release whereas external hypoxia alone does not. However, whilst we did not measure intracellular pH or lactic acid production, it is possible that anaerobic metabolism induced acidosis in our study. Nevertheless, fishes typically hyperventilate during hypoxia (Leite et al., 2009), likely compensating plasma pH (Maxime, Pichavant, Boeuf, & Nonnotte, 2000), and tightly regulate cardiac intracellular pH (Harter et al., 2014), so hypoxia is likely not associated with myocardial acidosis in fishes *in vivo*.

354 Whilst catecholamines were not spontaneously released from the myocardium during hypoxia, we saw that
355 tyramine increased the release of NA and increased twitch force. Previous work in other fishes has demonstrated
356 that tyramine has a positive inotropic effect during normoxia (Fänge & Östlund, 1954; Temma et al. 1989) thus
357 we extended this by demonstrating that the effect of tyramine is preserved in hypoxic myocardium. This
358 phenomenon must be neurally-mediated, given the inability for hypoxia per se to elicit catecholamine release in
359 the absence of tyramine, and thus release would have to be extrinsically triggered. This also appears likely because
360 tyramine has no inotropic effect on plaice myocardium (Falck et al., 1966; Cobb & Santer, 1973), which
361 characteristically lacks neuronal cardiac innervation (Laurent et al., 1983; Sandblom & Axelsson, 2011).
362
363 Figure 5 represents a cross-comparison of the protocols before and after the addition of endogenous and exogenous
364 catecholamines. This comparison was made at 0.6 Hz, which most closely corresponds to the hypoxic heart rate
365 in the related pacu (Leite et al., 2009). Although the relatively low sample size (n=4 for A and NA treated
366 preparations) in this non-repeated measures design precludes rigorous statistical analysis, it nonetheless provides
367 a useful descriptive tool. Given that, at this frequency, propranolol treated preparations exhibited a time-dependent
368 decline in force of 5 % between the force-frequency trials (Fig. 3), we conclude that both tyramine and NA were
369 responsible for a total restoration of force of approximately 12 %, whilst A increased force by about 20%. Because
370 our catecholamine measurements revealed that NA is the predominant catecholamine in the piranha ventricle, and
371 only NA was released into Ringer's solution in non-working preparations, it strongly suggests that tyramine exerts
372 its effects by releasing NA and not A. The concentration at the cell membrane (where the β-adrenergic receptors
373 are located) must be sufficiently high (i.e. at least 10 μM) to invoke the less pronounced NA-dependent response,
374 which is likely viable as the catecholamines are released locally from the myocardium. The finding that NA was
375 the predominant myocardial catecholamine and likely responsible for the action of tyramine is surprising given
376 that A was more effective at generating positive inotropic effects. The greater potency of A relative to NA,
377 however, is consistent with several previous studies in teleost myocardium (Fänge & Östlund, 1954; Falck et al.,
378 1966; Cobb & Santer, 1973; Holmgren, 1977). This also means that if A is humorally released only *in extremis*
379 (Perry et al., 2004) it may still provide an additional functional benefit to the heart.
380
381 *Force-frequency Relationship*
382 Piranhas exhibited the typical negative piscine force-frequency relationship (Shiels, Vornanen, & Farrell, 2002).
383 However, similar to other tropical fish, some of which exhibit flat force-frequency relationships (Shiels, Santiago,

& Galli, 2010), this relationship was not steep. If red-bellied piranhas develop a hypoxic bradycardia similar to the closely related pacu (approximately 60 to 36 beats min^{-1}; 1 Hz to 0.6 Hz), this would have a rather modest impact on contractile force (5% increase; based on the first force-frequency trial in untreated hypoxic preparations). This is much less significant than that we recently reported in the temperate European eel heart (*Anguilla anguilla,* up to 30% increase in twitch force with bradycardia) (Joyce et al., 2016). This blunted force-frequency relationship in tropical fish must limit the benefits of hypoxic bradycardia in the species most likely to require it, raising questions concerning its general functional significance. In this regard, other aspects of cardiac function, such as myocardial oxygenation or energy efficiency (Farrell, 2007), are likely more important than contractility *per se*. It is, however, interesting that the greatest effect of tyramine was observed at the low (bradycardic) frequencies during hypoxia but at higher frequencies (coincident with normoxic hearts rates) during normoxia (Fig. 3). It is therefore tempting to speculate that hypoxic bradycardia may serve to potentiate the adrenergic stimulation of the myocardium during hypoxia.

Conclusions

Our results show that the piranha ventricle contains substantial amounts of catecholamines, predominantly NA, which can be released by tyramine. This pool of NA can be engaged during hypoxia where it exerts a positive inotropic effect. Thus, our findings support the possible role for endogenous catecholamines to rescue myocardial performance during hypoxia, even in the absence of humoral catecholamine release (e.g. Perry et al., 2004). Future *in vivo* studies using relevant pharmacological tools are now required to ascertain whether and when this potential is realised.

Acknowledgements

We are sincerely grateful to Hans Gesser for allowing us unlimited access to equipment.

References

Bailey, J. R., Val, A. L., Almeida-Val, V., & Driedzic, W. R. (2011). Anoxic cardiac performance in Amazonian and north-temperate-zone teleosts. *Canadian Journal of Zoology*, 77, 683–689. doi: 10.1139/z99-019.

414 Cobb, J. L. S., Santer, R. M. (1973). Electrophysiology of cardiac function in teleosts: cholinergically
415 mediated inhibition and rebound excitation. *The Journal of Physiology*, 230, 561–573.
416 doi:10.1113/jphysiol.1973.sp010204.
417
418 Cros, C., Sallé, L., Warren, D. E., Shiels, H. A., & Brette, F. (2014). The calcium stored in the sarcoplasmic
419 reticulum acts as a safety mechanism in rainbow trout heart. *American Journal of Physiology. Regulatory,*
420 *Integrative and Comparative Physiology*, *307*(12), R1493–501. http://doi.org/10.1152/ajpregu.00127.2014
421
422 Donald, J., & Campbell, G. (1982). A comparative study of the adrenergic innervation of the teleost heart.
423 *Journal of Comparative Physiology*, *147*(1), 85–91. http://doi.org/10.1007/BF00689295
424
425
426 Gannon, B. J., & Burnstock, G. (1969). Excitatory adrenergic innervation of the fish heart. *Comparative*
427 *Biochemistry and Physiology*, *29*(2), 765–773. http://doi.org/10.1016/0010-406X(69)91627-2
428
429
430 Gaffney T. E., Morrow D. H., & Chidsey, C. A. (1962). The role of myocardial catecholamines in the
431 response to tyramine. *The Journal of Pharmacology and Experimental Therapeutics,* 137, 301–305.
432
433 Gesser H, Andresen P, Brams P, Sund-Laursen J. 1982. Inotropic effects of adrenaline on the anoxic or
434 hypercapnic myocardium of rainbow trout and eel. J Comp Physiol B 147:123–128.
435
436 Falck, B., Mecklenburg, C.V., Myhrberg, H., & Persson, H., (1966). Studies on Adrenergic and Cholinergic
437 Receptors in the Isolated Hearts of *Lampetra fluviatilis* (Cyclostomata) and *Pleuronectes platessa* (Teleostei).
438 *Acta Physiologica Scand,* 68, 64–71. doi:10.1111/j.1748-1716.1966.tb03403.
439
440 Farrell, A. P., & Smith, F. (2017). Cardiac Form, Function and Physiology. In A. K. Gamperl, T. E. Gillis,
441 A. P. Farrell, & C. J. Brauner (Eds.), *Fish Physiology* (Vol. 36, pp. 155–264). Academic Press.
442 http://doi.org/10.1016/bs.fp.2017.07.001
443
444 Fritsche, R., & Nilsson, S. (1990). Autonomic nervous control of blood pressure and heart rate during hypoxia
445 in the cod, *Gadus morhua*. *Journal of Comparative Physiology B*, 160, 287–292. doi: 10.1007/BF00302594.
446
447 Fujino, K., Yoshitake, T., Kehr, J., Nohta, H., & Yamaguchi, M., (2003). Simultaneous determination of 5
448 hydroxyindoles and catechols by high-performance liquid chromatography with fluorescence detection
449 following derivatization with benzylamine and 1,2-diphenylethylenediamine, *Journal of Chromatography A,*
450 1012, 169–177. doi:10.1016/S0021-9673(03)01180-4.
451

Fänge R., & Östlund E. (1954). The effects of adrenaline, noradrenaline, tyramine and other drugs on the isolated heart from marine vertebrates and a cephalopod (*Eledone cirrosa*). *Acta Zoologica*, 35, 289–305. doi: 10.1111/j.1463-6395.1954.tb00037.

Hanson LM, Obradovich S, Mouniargi J, & Farrell AP. (2006). The role of adrenergic stimulation in maintaining maximum cardiac performance in rainbow trout (Oncorhynchus mykiss) during hypoxia, hyperkalemia and acidosis at 10 degrees C. *Journal of Experimental Biology* 209, 2442–2451.

Harter, T. S., Shartau, R. B., Baker, D. W., Jackson, D. C., Val, A. L., & Brauner, C. J. (2014). Preferential intracellular pH regulation represents a general pattern of pH homeostasis during acid-base disturbances in the armoured catfish, Pterygoplichthys pardalis. *Journal of Comparative Physiology. B, Biochemical, Systemic, and Environmental Physiology*, *184*(6), 709–718. http://doi.org/10.1007/s00360-014-0838-8

Holmes, J. C., & Fowler, N. O. (1962). Cardiac effects of tyramine. *Circulation Research*, *11*, 364–369.

Huang, M. H., Friend, D. S., Sunday, M. E., Singh, K., Haley, K., Austen, K.F., Kelly, R.A., & Smith, T.W. (1996). An intrinsic adrenergic system in mammalian heart. *Journal of Clinical Investigation*, 98, 1298–1303. doi: 10.1172/JCI118916.

Iversen, N.K., Lauridsen, H., Huong, D.T.T., Nguyen, V.C., Gesser, H., Buchanan, R., Bayley, M., Pedersen, M., & Wang, T. (2013). Cardiovascular anatomy and cardiac function in the air-breathing swamp eel (*Monopterus albus*). *Comparative Biochemistry and Physiology. Molecular & Integrative Physiology,* 164, 171–180. doi: 10.1016/j.cbpa.2012.08.007.

Joyce, W., Gesser, H., Bayley, M., & Wang, T. (2015). Anoxia and Acidosis Tolerance of the Heart in an Air-Breathing Fish (*Pangasianodon hypophthalmus*). *Physiological and Biochemical Zoology,* 88, 648–659. doi: 10.1086/682701

Joyce, W., Simonsen, M., Gesser, H., & Wang, T. (2016). The effects of hypoxic bradycardia and extracellular HCO3(-)/CO2 on hypoxic performance in the eel heart. *The Journal of Experimental Biology*, *219*(Pt 3), 302–305. http://doi.org/10.1242/jeb.130971

Lameris, T. W., van den Meiracker, A. H., Boomsma, F., Alberts, G., de Zeeuw, S., Duncker, D. J., et al. (1999). Catecholamine handling in the porcine heart: a microdialysis approach. *American Journal of Physiology-Heart and Circulatory Physiology*, *277*(4), H1562–H1569. http://doi.org/10.1152/ajpheart.1999.277.4.H1562

Lameris, T. W., de Zeeuw, S., Alberts, G., Boomsma, F., Duncker, D. J., Verdouw, P. D., Veld, A. J. M,, van den Meiracker, A. H. (2000). Time Course and Mechanism of Myocardial Catecholamine Release During Transient Ischemia In Vivo. *Circulation*, *101*(22), 2645–2650. http://doi.org/10.1161/01.CIR.101.22.2645

Laurent, P., Holmgren, S., & Nilsson, S. (1983). Nervous and humoral control of the fish heart: Structure and function. *Comparative Biochemistry and Physiology A Comparative Physiology,* 76, 525–542. doi: 10.1016/0300-9629(83)90455-3

Leite, C.A.C., Taylor, E.W., Guerra, C.D.R., Florindo, L.H., Belão, T., & Rantin, F.T. (2009). The role of the vagus nerve in the generation of cardiorespiratory interactions in a neotropical fish, the pacu, *Piaractus mesopotamicus*. *Journal of Comparative Physiology, A* 195, 721–731. doi: 10.1007/s00359-009-0447-2

Maxime, V., Pichavant, K., Boeuf, G., & Nonnotte, G., (2000). Effects of hypoxia on respiratory physiology of turbot, *Scophthalmus maximus*. *Fish Physiology and Biochemistry,* 22, 51–59. doi:10.1023/A:1007829214826

Östlund, E. (1954) The distribution of catechol amines in lower animals and their effect on the heart. *Acta Physiologica Scandinavica Suppl,* 31:1–67.

Perry, S.F., Fritsche, R., Kinkead, R., & Nilsson, S. (1991) Control of Catecholamine Release In Vivo and In Situ in the Atlantic Cod (*Gadus morhua*) During Hypoxia. *Journal of Experimental Biology,* 155, 549–566.

Perry S.F., & Bernier, N.J. (1999) The acute humoral adrenergic stress response in fish: facts and fiction. *Aquaculture* 177, 285–295. doi: 10.1016/S0044-8486(99)00092-7

Perry, S.F., Reid, S.G., Gilmour, K.M., Boijink, C. L., Lopes, J.M., Milsom, W.K., & Rantin, F.T. (2004) A comparison of adrenergic stress responses in three tropical teleosts exposed to acute hypoxia. *American Journal of Physiology Regulatory, Integrative and Comparative Physiology,* 287, R188–R197. doi: 10.1152/ajpregu.00706.2003

Perry, S.F., Gilmour, K.M., Vulesevic, B., McNeill, B., Chew, S.F., & Ip, Y.K. (2005) Circulating Catecholamines and Cardiorespiratory Responses in Hypoxic Lungfish (*Protopterus dolloi*): A Comparison of Aquatic and Aerial Hypoxia. *Physiological and Biochemical Zoology,* 78, 325–334. doi: 10.1086/430041

Ristori M.T., & Laurent, P. (1989) Plasma catecholamines in rainbow trout (*Salmo gairdneri*) during hypoxia. *Experimental Biology*, 48, 285–290.

Sandblom, E., Axelsson, M., (2011). Autonomic control of circulation in fish: A comparative view. *Autonomic Neuroscience,* 165, 127–139. doi:10.1016/j.autneu.2011.08.006

Santer, R.M., Cobb, J.L.S. (1972) The fine structure of the heart of the teleost*, Pleuronectes platessa* L. *Zeitschrift Fur Zellforschung Und Mikroskopische Anatomie,* 131, 1–14. doi: 10.1007/BF00307196

Schömig, A., Dart, A.M., Dietz, R., Mayer, E., & Kübler, W. (1984) Release of endogenous catecholamines in the ischemic myocardium of the rat. Part A: Locally mediated release. *Circulation Research,* 55, 689–701. doi: 10.1161/01.RES.55.5.689

Schömig A, Fischer S, Kurz T, & Richardt, G. (1987) Nonexocytotic release of endogenous noradrenaline in the ischemic and anoxic rat heart: mechanism and metabolic requirements. *Circulation Research*, 60, 194–205. doi: 10.1161/01.RES.60.2.194

Shiels, H.A., Vornanen, M., Farrell, A.P. (2002) The force-frequency relationship in fish hearts - A review. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology,* 132, 811–26. doi: 10.1016/S1095-6433(02)00050-8

Shiels, H.A., Santiago, D.A., & Galli G.L.J. (2010) Hypercapnic Acidosis Reduces Contractile Function in the Ventricle of the Armored Catfish, *Pterygoplichthys pardalis*. *Physiological and Biochemical Zoology,* 83, 366–375. doi: 10.1086/644759

Stecyk, J. A. W., Larsen, B. C., & Nilsson, G. E. (2011). Intrinsic contractile properties of the crucian carp (Carassius carassius) heart during anoxic and acidotic stress. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology*, *301*(4), R1132–42. http://doi.org/10.1152/ajpregu.00372.2010

Stoyek, M. R., Croll, R. P., & Smith, F. M. (2015). Intrinsic and extrinsic innervation of the heart in zebrafish (*Danio rerio*). *Journal of Comparative Neurology*, *523*(11), 1683–1700. http://doi.org/10.1002/cne.23764

Temma, K., Kishi, H., Kitazawa, T., Kondo, H., Ohta, T., & Katano, Y. (1986). Beta-2 adrenergic receptors are not only for circulating catecholamines in ventricular muscles of carp heart (*Cyprinus carpio*). *Comparative Biochemistry and Physiology. C, Comparative Pharmacology and Toxicology*, *83*(2), 265–269.

Temma, K., Akamine, M., Shimizu, T., Kitazawa, T., Kondo, H., & Ohta, T. (1989) Histamine directly acts on β-adrenoceptors as well as H1-histaminergic receptors, and causes positive inotropic effects in isolated ventricular muscles of carp heart (*Cyprinus carpio*). *Comparative Biochemistry and Physiology Part C: Comparative Pharmacology,* 92, 143–148. doi: 10.1016/0742-8413(89)90217-X

von Euler U.S., & Fänge R (1961) Catecholamines in nerves and organs of *Myxine glutinosa*, *Squalus acanthias,* and *Gadus callarias. General and Comparative Endocrinology,* 1, 191–194. doi: 10.1016/0016-6480(61)90028-4

Vornanen, M. (1998). L-type Ca2+ current in fish cardiac myocytes: effects of thermal acclimation and beta-adrenergic stimulation. *The Journal of Experimental Biology*, *201*(4), 533–547.

Yamauchi, A., & Burnstock, G. (1968) An electron microscopic study on the innervation of the trout heart. *Journal of Comparative Neurology,* 132, 567–587. doi: 10.1002/cne.901320406

JEZ figure legends

Figure 1. Time–dependent effect of hypoxia on normalized twitch force (A), rate of contraction (B) and rate of 50% relaxation (C) in red-bellied piranha ventricle strips. All values were normalized to the state 30 min before hypoxia treatment. Circle symbols represent time-dependent controls maintained in normoxia (50% O2, 49% N2 and 1% CO2), square symbols are the preparations exposed to hypoxia (99% N2 and 1% CO2). Black symbols were untreated, clear symbols were incubated with propranolol (1 μM) for 30 min prior to the recording period and thereafter. Statistical analysis described in text. N=6. All values are mean ± SEM.

Figure 2. The effect of stimulation frequency on normalized twitch force (A, B), rate of contraction (C, D) and rate of 50% relaxation (E, F) in red-bellied piranha ventricle strips exposed to normoxia (circles) or hypoxia (squares) in the presence (clear symbols) or absence (black symbols) of propranolol before (A, C, E) and after (B, D, F) treatment with the catecholamine releasing agent tyramine (0.3 mM). Statistical analysis described in text. N=6. All values are mean ± SEM.

Figure 3. The change in normalized twitch force (A), rate of contraction (B) and rate of 50% relaxation (C) in red-bellied piranha ventricle strips at each stimulation frequency after the addition of tyramine (0.3 mM). Preparations were exposed to normoxia (circles) or hypoxia (squares) in the presence (clear symbols) or absence (black symbols) of propranolol. Statistical analysis described in text. N=6. All values are mean ± SEM.

Figure 4. The effect of exogenous catecholamines, adrenaline (black) or noradrenaline (clear), on the force-frequency relationship in red-bellied piranha ventricle strips during normoxia (circles) or hypoxia (squares). Statistical analysis described in text. N=5 for panels A and B and N=4 for panel C. All values are mean ± SEM.

Figure 5. The effect of tyramine (Tyr), noradrenaline (NA) or adrenaline (A) on normalized twitch force at 0.6 Hz in hypoxia exposed preparations. A) Before treatment (force-frequency trial (FF) 1) and after treatment (FF2) with Tyr, NA or A. B= Change in force between FF1 and FF2. N=4-6. All values are mean ± SEM.