Intrinsic contractile properties of the crucian carp (Carassius carassius) heart during anoxic and acidotic stress

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Abstract

The crucian carp (Carassius carassius) seems unique among vertebrates in its ability to maintain cardiac performance during prolonged anoxia. We investigated whether this phenomenon arises in part from a myocardium tolerant to severe acidosis or because the anoxic crucian carp heart may not experience a severe extracellular acidosis due to the fish’s ability to convert lactate to ethanol. Spontaneously contracting heart preparations from cold-acclimated (6-8°C) carp were exposed (at 6.5°C) to graded or ungraded levels of acidosis under normoxic or anoxic conditions and intrinsic contractile performance was assessed. Our results clearly show that the carp heart is tolerant of acidosis as long as oxygen is available. However, heart rate and contraction kinetics of anoxic hearts were severely impaired when extracellular pH was decreased below 7.4. Nevertheless, the crucian carp heart was capable of recovering intrinsic contractile performance upon reoxygenation regardless of the severity of the anoxic + acidotic insult. Finally, we show that increased adrenergic stimulation can ameliorate, to a degree, the negative effects of severe acidosis on the intrinsic contractile properties of the anoxic crucian carp heart. Combined, these findings indicate an avoidance of severe extracellular acidosis and adrenergic stimulation are two important factors protecting the intrinsic contractile properties of the crucian carp heart during prolonged anoxia, and thus likely facilitate the ability of the anoxic crucian carp to maintain cardiac pumping.

Keywords: adrenaline, acidosis, anoxia, chronotropy, inotropy, intrinsic contractile performance, heart rate
Introduction

Deprivation of ambient oxygen is quickly lethal to most vertebrates. However, some species such as freshwater turtles of the genera *Chrysemys*, *Trachemys* and *Chelydra* and the crucian carp (*Carassius carassius*) have evolved an extreme tolerance to anoxia, allowing them to overwinter in ice-covered ponds that become depleted of oxygen (14, 56). At the cold wintertime temperatures, their anoxic survival time extends to months. At 20 – 25°C, their anoxia tolerance is still spectacular compared to most vertebrates, but is reduced to hours or days.

What is especially striking about the crucian carp is its ability to survive anoxia in an ‘active’ metabolic state via an upregulation of glycolysis fuelled by enormous glycogen stores, although levels of glycolytic enzymes are suppressed after prolonged anoxia at cold temperature (see ref. (56) for review of the physiology of anoxic crucian carp). Notably, brain activity is maintained, but some aspects of central nervous system function are reversibly reduced during anoxia. Moreover, protein synthesis and mitosis continue during prolonged anoxia. Indeed, the crucian carp was found to continue spontaneous swimming activity during 5 h of anoxia at 9°C, albeit at a 50% lower level than in normoxia.

Correspondingly, anoxic crucian carp possess the seemingly unique ability among vertebrates to maintain cardiac activity at normoxic levels (41). Upon anoxia exposure, crucian carp exhibit an immediate and substantial bradycardia (41, 42, 54, 57) much like in other hypoxia-sensitive and hypoxia-tolerant fish (4, 36, 38). However, by 48 h of anoxia at 8°C, cardiac output, heart rate, cardiac power output and stroke volume are all returned to control normoxic levels, where they remain stable for at least 5 d (41).
The goal of the present study was to lend insight into how the crucian carp is capable of maintaining cardiac activity at normoxic levels during prolonged anoxia. In particular, we focussed on the elucidating the effects of acidosis on the intrinsic contractile properties of the crucian carp heart. Inevitably, continued anaerobic glycolysis leads to an accumulation of lactate and $\text{H}^+$. For vertebrate hearts, acidosis reduces contractile force and can induce arrhythmias, which dramatically decreases the ability of the heart to pump blood (22). Thus, for the crucian carp heart, a number of potential pH management strategies exist that could contribute to the maintained cardiac activity during anoxia. Firstly, the crucian carp heart could be tolerant to extracellular acidosis. For instance, the anoxia-tolerant freshwater turtle heart is more resilient to acidosis than the hearts of hypoxia-sensitive species (9). On the other hand, the crucian carp has evolved the exotic ability to convert $\text{H}^+$ and lactate into ethanol and $\text{CO}_2$, which are released into the surrounding water (34). This process presumably allows the fish to minimize the metabolic acid load during prolonged anoxia. For example, for a single $15^\circ\text{C}$ crucian carp exposed to 26 h of anoxia, the onset of anoxia involved accumulation of lactate in the plasma and a decrease of plasma pH from the normoxic level of $\sim7.7$ (51). However, within 1.5 h of anoxia exposure, and coincident with the commencement of ethanol production, plasma lactate reached a steady-state level of about 8 mmol l$^{-1}$ and plasma pH stabilized near 7.4 (51). In this regard, the crucian carp’s ability to maintain cardiac activity at normoxic levels during prolonged anoxia may not stem from a tolerance of acidosis, but rather to the avoidance of severe extracellular acidosis. Nevertheless, crucian carp plasma pH following days or weeks of anoxia at cold temperature remains unknown, and an avoidance of severe extracellular acidosis during anoxia does not necessarily preclude the crucian carp heart to be tolerant of severe acidosis. Finally, moderate acidosis can have a protective effect on the vertebrate heart (1, 19). Thus, a moderate
extracellular acidosis during anoxia could be of benefit to crucian carp cardiac performance during prolonged anoxia exposure.

Given the scarcity of reported values for crucian carp plasma pH during prolonged anoxia at cold temperature, as well as the various potential pH management strategies that could foreseeably contribute to the maintained cardiac activity of anoxic crucian carp, we investigated the intrinsic contractile responses of the crucian carp heart to acidotic stress. Our approach was to expose a spontaneously contracting whole heart preparation to graded or ungraded levels of acidosis (ranging between pH 7.8 to 7.0) under normoxic or anoxic conditions and simultaneously measure the intrinsic chronotropic and inotropic properties of the preparation. The exposure protocols were designed to assess the ability of the crucian carp heart to contract in face of, and recover from, acidotic stress of varying severity. Specifically, the experiments were designed to distinguish between two hypotheses: 1) the ability of the crucian carp heart to beat at normoxic levels during prolonged anoxia exposure is facilitated by a myocardium tolerating acidosis; or 2) the ability of the crucian carp heart to beat at normoxic levels during prolonged anoxia exposure is aided by an avoidance of severe extracellular acidosis stemming from ethanol production. For the latter, we hypothesized the intrinsic contractile properties of the crucian carp heart to be severely impaired at an extracellular pH below the previously reported anoxic level of 7.4 (51).

Additionally, given the presence of sympathetic cardiac control in the crucian carp during prolonged anoxia exposure (41) and the well established role of adrenaline in counteracting the negative inotropic and chronotropic effects of acidosis on vertebrate hearts (e.g., ref.(12) and references therein), we also examined if an augmented extracellular adrenaline concentration
modified the intrinsic contractile responses of the crucian carp heart to a combined anoxia and graded acidotic exposure.

**Materials and methods**

*Experimental animals and ethical approval*

Fifty-seven crucian carp (*Carassius carassius* L.) of both sexes and with a mean body mass of 48 ± 32 g (mean ± SD) were utilized. Carp were trapped in a local pond (Tjernsrud, Oslo municipality, Norway) in the September and October and held indoors for at least 3 months prior to experimentation in a 370 L tank supplied with 6-8°C, aerated and dechlorinated Oslo tap water. Photoperiod was held constant at 12h:12h L:D and the fish were fed daily with commercial carp food (Tetra Pond, Tetra Melle, Germany). All experimental procedures were performed in accordance with regulations of the Norwegian Animal Research Authority.

*Tissue preparation*

A spontaneous contracting whole heart preparation similar to that utilized previously to examine intrinsic contractile properties of the fish heart, including that of the crucian carp (16, 45, 48, 49, 55, 57) was used to investigate the effect of extracellular anoxia, acidosis and adrenaline on spontaneous heart rate and contractile properties of the crucian carp heart. Fish were anaesthetized with buffered tricaine methanesulphonate (MS-222, Sigma, Norway; 0.4 mol l⁻¹ MS-222 + 0.4 mol l⁻¹ NaHCO₃) until opercular movement ceased, weighed and injected with 1.0 ml kg⁻¹ of heparinised saline (100 IU ml⁻¹) via the caudal blood vessels. The fish was
decapitated and the heart, with intact atrium and sinus venosus to allow the sino-atrial node to generate spontaneous action potentials, was then gently excised via a midventral incision and placed in ice-cooled control normoxic physiological saline (see Saline solutions for saline composition). To aid the flow of saline through the contracting ventricle, the bulbus was carefully trimmed away. Then, braided silk suture threads (size 5-0) were affixed to the ventricle at its apex and near the ventricular-bulbar junction. One thread was subsequently fastened to a force displacement transducer (Harvard Isotonic Transducer 50-6360, Harvard Apparatus, USA) configured for auxotonic contractions. The other thread was attached to a fixed arm, and the preparation suspended in a 40 ml water-jacketed tissue bath containing control normoxic physiological saline. Temperature of the tissue bath was maintained at 6.5°C with a circulating water bath (Heto, InterMed, Denmark). The length of the mounted preparation was adjusted with a micrometer screw to produce ~90% of maximal contraction force to limit inter-preparation variation due to the chronotropic and inotropic effects of cardiac stretch (3). No further adjustment was made to the length of the preparation during the duration of the experiment. The preparation was then permitted 20 - 25 min to stabilize and to allow washout of any endogenous adrenergic substances. At the end of the 20 – 25 min stabilization period the control normoxic physiological saline was renewed and an experimental protocol initiated.

Saline solutions

Physiological saline composition was (in mmol l⁻¹): NaCl, 124.1; KCl, 3.1; MgSO₄ · 7 H₂O, 0.9; CaCl₂ · 2 H₂O, 2.5; D-glucose, 5.6; adrenaline, 0.001, as commonly utilized for freshwater fish cardiac experiments (15). D-glucose and adrenaline were added immediately prior to use. The
D-glucose concentration falls within the circulating levels reported for normoxic and anoxic goldfish (*Carassius auratus* (35)), and the adrenaline concentration reflects that of normoxic common carp (*Cyprinus carpio* (50)). Similar to previous studies examining the effect of anoxia on the fish heart (24, 25), a mix between N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic (TES) free acid and TES Na salt was used to achieve the required pH of 7.8 for the control normoxic and control anoxic saline, or pH 7.6, 7.4, 7.2 and 7.0 for the acidotic conditions. The respective concentrations of TES free acid and Na salt were (in mmol l⁻¹): 4.83 and 2.60 for pH 7.8; 5.97 and 2.02 for pH 7.6; 7.01 and 1.50 for pH 7.4; 7.88 and 1.07 for pH 7.2; 8.55 and 0.73 for pH 7.0. The normoxic pH of 7.8 at 6.5°C was calculated from the normoxic pH of 15°C crucian carp using alpha-stat correction for the normal change in blood pH with temperature (∆pK/dT of 0.016 pH units deg⁻¹), and the acidotic pH levels chosen to span the reported anoxic blood pH of 7.4 (51). In all instances, saline pH was confirmed prior to use (Metrohm 744 pH meter; Metrohm Ion Analysis, Switzerland).

For two reasons, a TES-buffered saline solution was utilized to manipulate H⁺ concentration *per se* rather than a bicarbonate and CO₂ system. Firstly, a TES-buffered system allowed us to isolate the effects of increased H⁺ concentration on crucian carp heart contractile properties while avoiding potential confounding effects of altered CO₂ and bicarbonate concentrations on fish heart cardiac contractility (8, 10). Secondly, to the best of our knowledge, circulating concentrations of CO₂ and bicarbonate have not been reported for normoxic or anoxic crucian carp and we wished to avoid potentially erroneous assumptions as to what these might be. In contrast, it is well reported that acidosis is largely of metabolic, and not respiratory, origin for anoxic crucian carp (35). Indeed, CO₂ is metabolically produced during anoxia in the ethanol producing biochemical pathway. However, the CO₂ is excreted to the ambient water (35).
Crucian carp exhibit an initial hyperventilation upon the onset of anoxia exposure and then down-regulate respiration rate to the pre-anoxic level for up to 5 d of anoxia (41). Nevertheless, it is important to note the absence of bicarbonate in the saline solutions could exaggerate the decline of intrinsic cardiac contractile properties in anoxia. Elevated bicarbonate has been shown to aid cardiac contractility during hypoxia exposure for common carp (Cyprinus carpio) and rainbow trout (Oncorhynchus mykiss) (8). Similarly, the possible, but to the best of our knowledge previously unexamined, changes in crucian carp blood composition with prolonged anoxia exposure (i.e., hyperkalemia, hypercalcemia, increased adrenaline concentration) was not examined in concert with the anoxic and acidic stressors. Such potential changes in blood composition could theoretically augment or diminish the effects of anoxia and acidosis on the intrinsic contractile properties of the carp heart reported here (e.g. (1, 26, 37)).

Normoxic conditions were achieved by bubbling the saline in the tissue bath with air. The crucian carp myocardium relies solely on luminal oxygen supply, and for the closely related goldfish, venous $P_{O2}$ is approximately 2 mmHg in normoxia (2). Thus for normoxia, air-, rather than 100% oxygen, was utilized to avoid potential detrimental effects of hyperoxia. To achieve anoxia, the bath saline was continuously bubbled with 99.99% N₂ and the top of the tissue bath was sealed except for a small hole allowing the silk thread to exit. Further, to ensure rapid anoxia exposure and to avoid a rise in bath saline oxygenation when changing anoxic saline solutions, refrigerated saline was bubbled with N₂ for at least 1.5 hours prior to use and a syringe was utilized to transfer the anoxic saline to the tissue bath via the drainage opening at the bottom. A low oxygen condition in the experimental chamber ($P_{O2} < 0.3 \text{ mmHg}$) was confirmed in preliminary experiments with an Oxi 340i oxygen electrode (WTW, Weilheim, Germany). The uniform exposure of all cardiac tissue to the saline solution was examined and confirmed in
preliminary experiments by visualizing with food dye the flow of saline through the atrium and ventricle.

*Experimental protocols*

Following the stabilization period, heart preparations were randomly assigned to either a control normoxic protocol or one of eight treatment protocols that were divided into two series. For the control normoxic protocol, hearts ($n = 6$) were maintained in control normoxic saline for 100 min, although the saline was renewed every 20 min. The first series of experimental protocols (#1 – 5) examined the ability of the crucian carp heart to function in face of a progressive acidic stress in normoxia or anoxia. Specifically, treatment protocols #1 – 4 were devised to test the hypothesis that crucian carp intrinsic cardiac contractile performance in anoxia is compromised below the reported anoxic extracellular pH of 7.4. Treatment protocol #5 was designed to test the hypothesis that the negative effects of an extracellular acidosis below pH 7.4 on the carp heart could be counteracted by an increased adrenergic stimulation. The five treatment protocols were conducted as follows.

1. *Normoxia + graded acidosis to pH 7.0*: Seven hearts were exposed to progressive acidosis in normoxia over 100 min. pH was lowered in steps of 0.2 units from 7.8 every 20 min.

2. *Anoxia at pH 7.8*: Eight hearts were exposed to 100 min of anoxic saline at pH 7.8. Anoxic saline was renewed every 20 min.

3. *Anoxia + graded acidosis to pH 7.0*: Nine hearts were exposed to progressive acidosis in anoxia over 100 min. pH was lowered in steps of 0.2 units from 7.8 every 20 min.
4. Anoxia + graded acidosis to a constant pH 7.4: Six hearts were exposed to a pH regime that mimicked the reported in vivo condition. pH was reduced to 7.6 after 20 min of anoxia and pH 7.4 after 40 min of anoxia, but was then maintained at the reported anoxic level of 7.4 for a total of 60 min, although the saline was renewed every 20 min.

5. Anoxia + graded acidosis to pH 7.0 with increased adrenergic stimulation: Six hearts were exposed to an identical protocol as #3, except that adrenaline concentration was increased to 25 nmol l⁻¹ during the pH 7.2 and pH 7.0 exposures. The adrenaline concentration of 25 nmol l⁻¹ falls within the concentration of circulating adrenaline and noradrenaline in severely hypoxic common carp (50).

A second series of treatment protocols examined the ability of the crucian carp heart to recover upon reoxygenation after a combined anoxic + acidotic insult of varying severity. Again, the pH levels examined were selected to span the reported anoxic extracellular pH of 7.4. However, unlike the first series of treatment protocols where extracellular pH was progressively decreased, the acidotic insults in the second series of treatment protocols were ungraded. The protocols were as follows.

6. Anoxia at pH 7.8 followed by reoxygenation: Five hearts were exposed to 40 min of anoxia at pH 7.8 and subsequently reoxygenated with control normoxic saline for 40 min. Anoxic + acidotic and normoxic saline solutions were renewed every 20 min.

7. Anoxia at pH 7.4 followed by reoxygenation: Five hearts were exposed to 40 min of anoxia at pH 7.4 and subsequently reoxygenated with control normoxic saline for 40 min. Anoxic + acidotic and normoxic saline solutions were renewed every 20 min.
7. Anoxia at pH 7.0 followed by reoxygenation: Five hearts were exposed to 40 min of anoxia at pH 7.0 and subsequently reoxygenated with control normoxic saline for 40 min. Anoxic + acidotic and normoxic saline solutions were renewed every 20 min.

For both series of treatment protocols, the exposure time for each saline condition was chosen to obtain an effective balance between reaching new steady state and maintaining tissue integrity for the duration of the experiments. Further, for the first series of treatment protocols, the 20 min time period was based on previous studies reporting that, if present, inotropic and chronotropic responses of vertebrate myocardium to anoxic and/or acidotic challenges occurs within approximately 5 min to 15 min of exposure (9, 12, 20, 24, 58). The renewal of saline solutions every 20 min was also conducted to ensure continued viability of the photosensitive adrenaline.

Data analysis and statistics

The signal from the force transducer was continuously recorded at 100 Hz with a Powerlab 4/20 data acquisition system in conjunction with Chart software (AD Instruments, Colorado Springs, CO, USA). The force transducer was powered by the Powerlab 4/20 and the output signal was of sufficient strength such that a pre-amplifier was not required. The last 3 min of each 20 min exposure period was analyzed offline for the determination of spontaneous heart rate ($f_{HR}$), maximum contractile force ($F_{\text{max}}$; expressed as N g$^{-1}$ of tissue wet mass), time-to-peak force (TPT) and time-to-half relaxation from peak force ($T_{1/2R}$). TPT and $T_{1/2R}$ were calculated to characterize the duration of the contraction and relaxation phases of the cardiac cycle.
Spontaneous $f_{ti}$ was determined from the peak-to-peak intervals of the contraction trace and $F_{\text{max}}$, TPT and $T_{1/2R}$ were averaged from 10 randomly selected contractions.

Mean values ± S.E.M are presented at each sample time. For the first series of treatment protocols, two-way repeated measures (RM) analysis of variance (ANOVA) were used to compare measured parameters between treatment protocols. To test for an effect of graded acidosis during normoxia, normoxic hearts exposed to progressive acidosis (treatment protocol #1) were compared to the control normoxic hearts. To test for an effect of anoxia, hearts exposed anoxia at pH 7.8 (treatment protocol #2) were compared to control normoxic hearts. To determine if the severity of acidosis affected contractile properties of anoxic hearts, the hearts exposed to anoxia at pH 7.8 (treatment protocol #2) were designated the ‘control’ condition and were compared to the anoxic hearts exposed to progressive acidosis to pH 7.0 (treatment protocol #3), as well as to the anoxic hearts exposed to the simulated in vivo condition (treatment protocol #4). To test for effects of increased adrenergic stimulation, the anoxic hearts exposed to progressive acidosis to pH 7.0 with increased adrenaline concentration at pH 7.2 and 7.0 (treatment protocol #5) were compared to both the hearts exposed to anoxia at pH 7.8 (treatment protocol #2) as well as the anoxic hearts exposed to progressive acidosis to pH 7.0 (treatment protocol #3). For the second series of treatment protocols, a one-way ANOVA was utilized to compare cardiac contractile parameters among the three experimental groups and comparable control normoxic hearts at 0 min (control normoxia), 40 min of anoxia + acidosis exposure and 40 min of reoxygenation. In all instances, $P<0.05$ was used as the level for significance and multiple comparisons were performed using a Holm-Sidak post-test.
Results

Stability of the heart preparation

Control normoxic hearts remained viable throughout the duration of the experiment. No statistically significant changes in $f_H$, $F_{\text{max}}$, TPT or $T_{1/2R}$ occurred over the 100 min exposure to normoxic saline at pH 7.8 (Fig. 1A-H).

Effect of graded acidosis in normoxia

The heart preparations were tolerant to extracellular acidosis when oxygen was available. No statistically significant changes in $f_H$, $F_{\text{max}}$, TPT, $T_{1/2R}$ occurred when pH was decreased from 7.8 to pH 7.0 in normoxia (Fig. 1A-E). In fact, the intrinsic contractile performance of the normoxic, progressively acidotic hearts did not differ from that of the control normoxic hearts.

Effect of anoxia

Intrinsic cardiac contractile properties were reduced in response to anoxia exposure at a constant pH of 7.8 (Fig. 1E-H). After 60 min of anoxia, $f_H$ and $F_{\text{max}}$ were reduced by 39% and 38%, respectively (Fig. 1E-F). Following this initial decrease, no further statistically significant alterations occurred, although only $f_H$ appeared to reach a new “steady-state”. Simultaneously, TPT and $T_{1/2R}$ increased by 14% and 64%, respectively, before stabilizing (Fig. 1G-H).

Combined effects of anoxia and acidosis
Anoxic hearts exposed to graded acidosis to pH 7.0 (treatment protocol #3) exhibited patterns of change in $f_{hi}$, $F_{max}$, TPT and $T_{1/2R}$ similar to the “anoxic control” hearts (treatment protocol #2) as extracellular pH was decreased in a step-wise manner to 7.4 (Fig. 2A-D). However, when pH was reduced to 7.2 and subsequently 7.0 (i.e., below the reported anoxic pH of 7.4), $f_{hi}$, TPT and $T_{1/2R}$ were considerably slowed compared to the anoxic hearts maintained at pH 7.8 (Fig. 2A, C and D). Thus, crucian carp heart preparations exposed to a combination of anoxia and extracellular pH below 7.4 did not beat as frequently or contract and relax as quickly as anoxic hearts at higher extracellular pH. In contrast, the magnitude of decrease of $F_{max}$ with anoxia remained similar regardless if pH was maintained at 7.8 or progressively reduced to pH 7.0 (Fig. 2B).

To exclude the possibility that the slower $f_{hi}$ and contraction kinetics of anoxic hearts exposed to pH 7.2 and 7.0 arose from a time-dependent degradation of the preparations due to prior acidosis exposure, a group of hearts was exposed to anoxia and graded acidosis in a manner that mimicked the supposed *in vivo* situation, where pH initially decreases, but then stabilizes near pH 7.4 (treatment protocol #4; Fig. 2E-H). Hearts exposed to the simulated *in vivo* condition displayed identical intrinsic contractile properties to the anoxic hearts maintained at pH 7.8. This finding confirmed that intrinsic contractile performance of the anoxic crucian carp heart is severely affected by a fall in pH below the reported anoxic level of 7.4.

*Effect of adrenaline during anoxia and severe acidosis*

Augmented adrenaline concentration ameliorated some of the negative effects of severely decreased pH on the intrinsic contractile properties of the anoxic crucian carp heart. In
particular, when extracellular adrenaline was increased from 1 to 25 nM at pH 7.2 and 7.0, $f_{II}$ was no longer significantly lower than the $f_{II}$ of “anoxic control” hearts (compare Fig. 2A and I). Similarly, the slowed TPT and $T_{1/2R}$ at pH 7.2 (Fig. 2C and D) were absent with increased adrenaline (Fig. 2K and L). At pH 7.0, the heightened adrenaline concentration partially counteracted the decreased TPT and $T_{1/2R}$ (Fig. 2K and L). TPT and $T_{1/2R}$ were significantly quicker than for hearts exposed to severe acidosis at the tonic level of adrenergic stimulation.

Nevertheless, augmented adrenaline did not completely offset the negative effects of severe acidosis on $f_{II}$ and contraction kinetics. Particularly, even though $f_{II}$ was elevated to a level statistically similar to that of the “anoxic control” hearts (Fig. 2I), $f_{II}$ was not significantly increased above the $f_{II}$ of the severely acidotic hearts. Similarly, at pH 7.2, only TPT, and not $T_{1/2R}$, was statistically significantly shortened compared to the hearts at the tonic level of adrenergic stimulation (Fig. 2K). Finally, at pH 7.0, both TPT and $T_{1/2R}$ remained statistically significantly slower than for the “anoxic control” hearts, despite the contraction kinetics being statistically significantly faster than the comparable severely acidotic hearts at the tonic level of adrenergic stimulation.

**Effects of ungraded acidosis and reoxygenation**

Like for the anoxic hearts exposed to progressive acidosis, exposure of spontaneously contracting crucian carp heart preparations to an immediate decrease in pH in conjunction with anoxia resulted in decreased intrinsic contractile performance (Fig. 3). Quantitatively, the reductions of intrinsic cardiac contractile properties with an immediate exposure to anoxia at pH 7.8, 7.4 or 7.0 were similar in magnitude to the depression of activity displayed by the hearts.
exposed to progressive acidosis in anoxia once the particular pH was reached. In particular, $f_H$ was reduced by ~30-40% when pH was 7.8 and 7.4 in anoxia, and by 68% when pH was 7.0 in anoxia, regardless if the fall in pH was achieved gradually or immediately. Thus, like in the first series of experimental treatments, the hearts immediately exposed to pH 7.0 in anoxia generally performed worse than hearts immediately exposed to pH 7.8 or pH 7.4 in anoxia. Most notably, $f_H$ (Fig. 3A) and TPT (Fig. 3C) at pH 7.0 were significantly slowed compared to comparable hearts at pH 7.8 and 7.4. However, the magnitude of decrease in $F_{\text{max}}$ with the ungraded anoxia + acidosis exposures was independent of the severity of acidosis (Fig. 3B), like in the first series of treatment protocols (Fig. 2).

Upon reoxygenation, the crucian carp heart was able to recover intrinsic contractile performance (Fig. 3). No statistically significant differences in $f_H$, $F_{\text{max}}$, TPT or $T_{1/2R}$ from comparable control normoxic hearts were apparent following 40 min of reoxygenation, regardless of the severity of the preceding anoxic + acidotic stress.

**Discussion**

Our objective was to better understand how the crucian carp maintains cardiac activity at normoxic levels during prolonged anoxia. The results suggest this extraordinary cardiac capability is aided in part by the ability of the anoxic crucian carp to turn H$^+$ and lactate into ethanol, thereby sheltering the intrinsic contractile properties of the heart from the significant metabolic acid load that would otherwise accompany a prolonged reliance on anaerobic metabolism. Specifically, the intrinsic contractile properties of spontaneously contracting crucian carp heart preparations exposed to anoxia and extracellular pH between 7.8 and 7.4 did
not differ from hearts exposed to anoxia and maintained at normoxic pH. In contrast, intrinsic contractile properties of anoxic hearts were severely compromised when extracellular pH was decreased below the reported \textit{in vivo} anoxic level of 7.4. This phenomenon occurred regardless of whether the crucian carp hearts were exposed to acidosis progressively, as they would be \textit{in vivo} (Fig. 2), or immediately (Fig. 3). Nevertheless, despite the seemingly detrimental effects of severe acidosis on anoxic crucian carp intrinsic cardiac contractile properties, the crucian carp heart was capable of recovering intrinsic contractile performance to control normoxic levels upon reoxygenation (Fig. 3). This finding indicates the anoxic crucian carp heart was not permanently damaged by severe acidosis. Finally, our experiments indicate that sympathetic cardiac stimulation and/or circulating catecholamines provide an additional layer of protection against the negative effects of a severely decreased extracellular pH on the intrinsic contractile properties of the crucian carp heart. Thus, the avoidance of severe extracellular acidosis and adrenergic stimulation are two important factors likely facilitating the ability of the crucian carp to maintain cardiac pumping during prolonged anoxia exposure.

\textit{Critique of Methods}

The small size of the experimental animals available for the present study precluded the use of an \textit{in situ} perfused heart preparation and necessitated the use of a spontaneously contracting whole heart preparation to simultaneously assess the chronotropic and inotropic effects of anoxia and acidosis on crucian carp cardiac function. Although the spontaneously contracting whole heart preparation does not provide information on the functional performance of the heart (with the exception of $f_{hi}$) it permits quantification of cardiac contraction parameters
such as TPT and $T_{1/2}$, which ultimately underlie functional performance. Indeed, numerous qualities of the preparation support its physiological relevance despite: i) the possibility that the absence of pumping against a physiological output pressure could lead to underestimation of the reduction of contractile performance in response to anoxic and acidotic stress; and ii) the mounting the entire heart for measurement of force production means all the elastic components present in the heart will reduce the force measured during contraction.

Importantly, the heart preparation did not display any degradation of intrinsic contractile properties throughout the 100 min control normoxic protocol (Fig. 1A-D). Given that intrinsic contractile performance decreased with anoxia (Fig. 1E-H), the maintained activity of the preparations under control normoxic conditions indicates the control normoxic hearts were not oxygen-limited and viable for the duration of experimental protocols.

As importantly, the heart preparation contracted at rates comparable to the in vivo intrinsic rate under both normoxic and anoxic conditions. In normoxia, spontaneous $f_H$ ranged between 18 and 21 min$^{-1}$ (Figs. 1-3), thus closely matching the previously reported in vivo intrinsic $f_H$ of 17.1 min$^{-1}$ for 8°C crucian carp (41). Spontaneous $f_H$ during normoxia in the present study was, however, greater than reported previously for 5°C- and 15°C-acclimated crucian carp heart preparations measured between 5°C and 10°C (16). The discrepancy may reflect differences in saline composition between studies. Namely, a lower K$^+$ (3.1 vs. 5 mmol l$^{-1}$) and greater tonic adrenaline (1 vs. 0 nmol l$^{-1}$) concentration in the current study. In anoxia, spontaneous $f_H$ of hearts exposed to pH 7.8 or 7.4 stabilized between 11 and 14 min$^{-1}$ (Figs. 1E, 2A, 2E and 3A), rates similar to the previously reported in vivo intrinsic $f_H$ of 13.7 min$^{-1}$ for 5 d anoxic crucian carp at 8°C (41). Thus, in terms of contraction frequency, the spontaneously
contracting heart preparation was performing similarly to the *in vivo* intrinsic condition under both normoxic and anoxic conditions.

Finally, the stabilization of $f_{\text{H}}$, TPT and $T_{1/2R}$ in anoxia (Fig. 1E, G and H) argues against the slowed contraction rate and kinetics arising from a gradual deterioration of the preparation. Further evidence against the depressed contractile state of the heart preparation in anoxia being due to deterioration of the preparation is provided by the fact all contractile parameters, including $F_{\text{max}}$, which unlike $f_{\text{H}}$, TPT and $T_{1/2R}$ continuously decreased during anoxia exposure (Fig. 1F), returned to control normoxic levels upon reoxygenation (Fig. 3).

Cumulatively, the very similar spontaneous $f_{\text{H}}$ of the preparation to the *in vivo* intrinsic $f_{\text{H}}$ in normoxia and anoxia, the stabilization of contractile performance during anoxia exposure at pH 7.8 and 7.4, and the ability of the preparation to recovery contractile performance upon reoxygenation, signifies that the findings of the present study can be extended to the *in vivo* condition and validates the designation of the hearts exposed to anoxia at pH 7.8 (treatment protocol #2) as the ‘control’ condition, to which the other treatment protocols can be compared (Fig. 2).

*Acidosis, anoxia and the crucian carp heart*

In general, anoxia and extracellular acidosis negatively affect the vertebrate heart. The negative effects of anoxia arise from an inhibition of excitation-contraction coupling and contractile proteins (17), an elevation of intracellular inorganic phosphate that decreases Ca$^{2+}$ sensitivity of the myofilament (9), and modification of myocardial action potential shape (44). Extracellular acidosis causes a secondary decline in intracellular pH, which inhibits excitation-contraction
coupling by reducing the magnitude of Ca$^{2+}$ entering cardiomyocytes and competitively hindering calcium-troponin binding (9, 23, 59). Additionally, extracellular acidosis modifies action potential shape (40) and inhibits spontaneous firing of pacemaker cells (31). Moreover, when combined, anoxia and acidosis can act synergistically to depress cardiac function (58).

However, inter- and intra-specific tolerances to anoxia and acidosis do exist among vertebrate hearts. The flounder (*Pleuronectes flesus*) heart exhibits a biphasic response to graded hypercapnic acidosis down to pH 7.0, recovering contractility to near control levels with continued exposure (10). Similarly, the European eel (*Anguilla anguilla*) can maintain cardiac output during a graded hypercapnic acidosis down to an arterial pH of 7.2 (18) and maximal *in situ* cardiac performance of the armoured catfish (*Pterygoplichthys pardalis*) is not compromised by hypercapnic acidosis until extracellular pH is decreased to 7.1 (11). For the freshwater turtles, cold-acclimation preconditions the heart for anoxia and acidosis (26, 37), and hearts from anoxia-tolerant species are more tolerant to, and recover better from, combined anoxia and acidosis than those from hypoxia-sensitive species (9).

The results from the present study indicate that the cold-acclimated crucian carp heart is extremely tolerant of extracellular acidosis as long as oxygen is available. No degradation of intrinsic cardiac contractile properties occurred with progressive acidosis from pH 7.8 to 7.0 in normoxia (Fig. 1A-D). For mammalian hearts, defence against intracellular acidosis is based on cellular ion flux pathways and intracellular buffering by HCO$_3^-$ and non-HCO$_3^-$ buffers (52). Briefly, during persistent acidosis, intracellular H$^+$ is extruded through the Na$^+$-H$^+$-exchanger (NHE). The consequent increase in intracellular Na$^+$ (which also occurs due to a Na$^+$-HCO$_3^-$ symport) generates an increase in intracellular Ca$^{2+}$ via reverse-mode functioning of the Na$^+$-Ca$^{2+}$-exchanger (NCX). The increased intracellular Ca$^{2+}$ serves to directly recover contractile
force. Further, the increased intracellular Ca\textsuperscript{2+} influx leads to a progressive loading of the sarcoplasmic reticulum (SR) with Ca\textsuperscript{2+}, which is released in subsequent cardiac cycles, dramatically increasing contractile strength. For ectothermic vertebrate hearts, including that of the crucian carp, the cellular ion flux pathways initiated in response to acidosis have not been thoroughly examined (reviewed by (39)). However, it is known that excitation-contraction coupling in crucian carp cardiomyocytes is almost exclusively dependent upon transsarcolemmal Ca\textsuperscript{2+} influx (via L-type Ca\textsuperscript{2+} channels and the NCX) and virtually independent of SR Ca\textsuperscript{2+} release (47). Thus, the NCX could be involved in maintaining contractility during acidosis exposure in the crucian carp. Interestingly though, for hearts of toad (*Bufo arenarum*) and painted turtle (*Chrysemys picta belli*), the NHE does not seem to play a critical role in the recovery from acidosis (30, 32). The relative importance of intracellular buffers, the NHE and the Na\textsuperscript{+}-HCO\textsubscript{3}\textsuperscript{-} symport in defending against normoxic acidosis in the crucian carp heart remains to be studied.

Crucian carp hearts exposed to anoxia suppressed intrinsic cardiac contractile properties (Fig. 1E-H). *In vivo*, the immediate cardiac response of crucian carp to anoxia has been attributed to a strong, cholinergic-mediated bradycardia (41, 57). The present findings indicate that an intrinsic cardiac component may also contribute to this initial suppression of cardiac performance. Most strikingly, spontaneous $f_{\text{intrinsic}}$ of anoxic hearts exposed to pH 7.8 and 7.4 (11 - 14 min\textsuperscript{-1}; Figs. 1E, 2A, 2E and 3A) closely matched the previously reported *in vivo* intrinsic $f_{\text{intrinsic}}$ of 13.7 min\textsuperscript{-1} for 5 d anoxic crucian carp at 8ºC (41). Similarly, in previous study, crucian carp hearts poisoned with cyanide and stimulated to contract at a constant rate quickly exhibited contractile failure, whereas those poisoned, but allowed to beat spontaneously, exhibited bradycardia and continued to beat for a prolonged time period (57). Combined, the past and present findings suggest that the crucian carp heart, when isolated from nervous input, reduces its
energy demands, likely to match ATP consumption with glycolytic ATP production. In the context of the present study, the energy savings gained from a reduced workload may also serve to protect the heart from intracellular acidosis, allowing more energy to be channelled to ion-pumps and Ca\textsuperscript{2+} mobilization.

Concurrent with the reduced \( f_{H} \) during anoxia exposure, \( F_{\text{max}} \) was reduced, and TPT and \( T_{1/2R} \) slowed (Fig. 1G and H). These reductions in contractile performance likely arose from the well-described negative effects of oxygen deprivation on cardiac contraction. Namely, an inhibition of excitation-contraction coupling and contractile proteins (17), an elevation of intracellular inorganic phosphate that decreases Ca\textsuperscript{2+} sensitivity of the myofilament (9), and modification of myocardial action potential shape (44). For mammalian skeletal muscle, slowed TPT reflects a reduction in the amplitude of the intracellular Ca\textsuperscript{2+} transient required for muscle contraction, whereas a slowed \( T_{1/2R} \) reflects: i) an increase in the duration of the intracellular Ca\textsuperscript{2+} transient due to a reduced rate of Ca\textsuperscript{2+} reuptake by SR pumps; and ii) a slowed actin-myosin cross-bridge detachment rate (6). For the anoxic crucian carp heart, it is unlikely the slowed \( T_{1/2R} \) arose from a reduced rate of Ca\textsuperscript{2+} reuptake by SR pumps because the crucian carp heart does not rely significantly on SR Ca\textsuperscript{2+} stores for cardiac contraction (47). Therefore, the slowed \( T_{1/2R} \) suggests Ca\textsuperscript{2+} extrusion across the sarcolemma is negatively affected by anoxia exposure. Whether the NCX and/or the sarcolemmal Ca\textsuperscript{2+}-ATPase is inhibited remains to be investigated.

\textit{In vivo}, crucian carp \( f_{H} \), cardiac output, stroke volume and cardiac power output all return to control normoxic levels by approximately 48 h after the onset of anoxia exposure (41). Clearly, the spontaneously contracting heart preparations utilized in the present study did not exhibit a recovery of intrinsic contractile properties during the anoxia and acidosis exposures (Figs. 1, 2 and 3). Rather, \( f_{H} \), TPT and \( T_{1/2R} \) stabilized at reduced levels (Fig. 1E, G and H).
However, the discrepancy is not unexpected given the relatively short duration of the *in vitro* experiment and absence of nervous input. More importantly, the stabilization of spontaneous $f_H$ at a rate similar to the *in vivo* intrinsic $f_H$ reported for 5 d anoxic crucian carp at 8°C (41) suggests that once a new intrinsic “steady-state” is achieved upon anoxia exposure, it is maintained for a prolonged period. A similar phenomenon has been reported for the cold, anoxic turtle heart (43). Thus, the close matching of *in vitro* and *in vivo* $f_H$ in anoxia suggests that mechanisms extrinsic to the crucian carp heart likely account for the increase in $f_H$ displayed by anoxic crucian carp *in vivo*. In depth speculation on the numerous possibilities is beyond the scope of the present study, which was to investigate acidosis and adrenergic stimulation, which are only two of the many potential factors, influencing crucian carp intrinsic cardiac performance during anoxia.

When exposed to acidosis during anoxia at a tonic adrenaline concentration, the cold-acclimated crucian carp heart was capable of coping with extracellular acidosis down to pH 7.4 (Figs. 2A-H and 3). This result is consistent with the previous report that the crucian carp survives prolonged anoxia with an extracellular pH near 7.4 (51). However, when extracellular pH was decreased below pH 7.4, intrinsic contractile properties of the crucian carp heart were severely compromised (Figs. 2A-D and 3). In nature, such reduced intrinsic contractile performance during anoxia may ultimately hinder the supply of nutrients to the brain and body as well as the circulatory requirements for waste transport. For example, the maintained cardiac pumping of anoxic crucian carp has been has been proposed (5, 41) to be essential for shuttling ethanol to the gills for excretion and for distributing sufficient amounts of glucose from the crucian carp’s large liver glycogen stores (13) to metabolically active tissues. Thus, the seemingly unique ability of the crucian carp heart to perform at normoxic levels during
prolonged anoxia exposure is not due to the myocardium displaying an extraordinary tolerance of acidosis, but rather appears to be permitted in part by the avoidance of severe extracellular acidosis stemming from ethanol production, which shelters the heart from the otherwise debilitating effects of severe acidosis on its intrinsic contractile properties.

The mechanisms underlying the reduction of anoxic crucian carp heart contractile performance when extracellular pH falls below 7.4 remain to be elucidated, but insight may be gained from the anoxia-tolerant turtle heart. Defence of intracellular pH in the turtle heart is less in the anoxic than normoxic state, a phenomenon hypothesized to be due to the controlled down-regulation of a Na$^+$ and HCO$_3^-$-dependent transporter as part of a greater strategy of metabolic suppression (32). Given the stark difference in acidosis tolerance between the normoxic and anoxic crucian carp heart (Figs. 1A-D and 2A-D), it is foreseeable that a suppression of acid-base regulatory ion transporters also occurs in the anoxic crucian carp heart. It may be that the degree of down-regulation represents a balance between reducing energy demands and maintaining the minimum level of activity to defend intracellular pH against the normal anoxic extracellular pH of 7.4. In this regard, it is interesting to note the magnitude of disruption in crucian carp heart contractile properties was largely proportional to the severity of extracellular pH, regardless if the acidosis was progressive or immediate (see Effects of ungraded acidosis and reoxygenation). Moreover, upon reoxygenation, the contractile properties of the crucian carp heart largely recovered to control normoxic levels, regardless of the preceding severity of the anoxic + acidotic insult (Fig. 3). The recovery indicates that the anoxic crucian carp heart was not permanently damaged by severe acidosis. Clearly, future studies measuring intracellular pH, as well as the expression and regulation of acid-base regulatory proteins at various extracellular pH in normoxia, anoxia and upon reoxygenation are needed to elucidate the
mechanisms underlying the reduction of anoxic crucian carp heart contractile performance when extracellular pH falls below 7.4.

The importance of adrenergic stimulation

In addition to cellular strategies, ectothermic vertebrates may also employ humoral means of protecting cardiac performance during anoxic and acidotic insults. Rainbow trout and American eel (*Anguilla rostrata*) release catecholamines into the circulation when hypoxic (27). Likewise, the catecholamine store in crucian carp chromaffin tissue is depleted during anoxia exposure (21). For teleost and turtle species, increased adrenergic stimulation protects the myocardium from the adverse effects of hypoxia- and exercise-induced acidosis (e.g., (12) and references therein) by increasing myocardial Ca\(^{2+}\) influx through sarcolemmal L-type channels, thereby counteracting the acidotic impairment of calcium-troponin binding (7, 28, 33, 53), and by increasing pacemaker self-excitation rate (46). Further, for the mammalian heart, adrenergic stimulation increases cross-bridge cycling rate (29).

In the present study, increased adrenergic stimulation partially ameliorated the compromised contractile properties of the anoxic crucian carp heart that occurred with exposure to pH 7.2, as well as the negative chronotropic effects that occurred with exposure to pH 7.0 (Fig. 2I-L). The role of adrenaline in protecting inotropic and chronotropic properties of the anoxic crucian carp heart corroborates the previous finding that pharmacological blockade of β-adrenoreceptors in anoxic crucian carp decreased $f_{IH}$ (41) and signifies redundancy in the defence strategy of the anoxic crucian carp to cardiac acidosis. Nevertheless, the rather moderate effects of adrenergic stimulation, as evidenced by the incomplete reversal of the negative effects of
severe acidosis on crucian carp (Fig. 2I-L), are consistent with recent findings that β-adrenergic signalling in isolated crucian carp cardiac tissues is far from potent (55).

Perspectives and significance

Survival of all vertebrates is dependent on uninterrupted cardiac function. Consequently, most vertebrates die within minutes when deprived of molecular oxygen in part because the heart requires a continuous supply of oxygen. Findings from the present study suggest that for the crucian carp, one of the champions of anoxia survival, the prevention of severe acidosis through ethanol production may be an important key underlying the maintenance of anoxic cardiac activity, and thus survival (provided cardiac glycolytic flux rate can supply ATP demands). Consequently, future research into adaptive mechanisms of anoxic cardiac tissue function should perhaps consider acidosis as being more detrimental in the long-term than anoxia per se. Currently, cellular investigations of the responses and defence strategies of anoxia-tolerant vertebrate hearts to acidosis are extremely limited, but hold promise to be an ideal avenue for future research aimed at understanding why some vertebrate hearts quickly die, whereas others can survive and continue to beat in the complete absence of oxygen.

Acknowledgements

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Figure Legend

**Fig. 1.** Comparison of the intrinsic contractile properties of 6.5°C spontaneously contracting crucian carp heart preparations exposed to: (A-D) control normoxia at pH 7.8 (n = 6) and graded acidosis from pH 7.8 to 7.0 in normoxia (n = 7); (E-H) control normoxia at pH 7.8 (n = 6) and anoxia at pH 7.8 (n = 8). Note the indication of extracellular pH on the x-axis is relevant only for the solid black symbols. Dissimilar letters indicate significant differences (P<0.05) among time points within an experimental group and an asterisk indicates a significant difference (P<0.05) between experimental groups at a specific time point (two-way RM ANOVA and Holm-Sidak post-test). Values are means ± SE.

**Fig. 2.** Comparison of the intrinsic contractile properties of 6.5°C spontaneously contracting crucian carp heart preparations exposed to: (A-D) anoxia at pH 7.8 (n = 8) and graded acidosis from pH 7.8 to 7.0 in anoxia (n = 9); (E-H) anoxia at pH 7.8 (n = 8) and graded acidosis limited to pH 7.4 in anoxia (n = 6); (I-L) anoxia at pH 7.8 (n = 8) and graded acidosis from pH 7.8 to 7.0 in anoxia with increased adrenaline (ADR; 25 nmol l⁻¹) at pH 7.2 and 7.0 (n = 6). Please note for panels I-L, the data from hearts exposed to graded acidosis from pH 7.8 to 7.0 in anoxia (black triangles) is once again depicted. For all panels, an asterisk indicates a significant difference (P<0.05) between the anoxic hearts at pH 7.8 (grey triangles) and the other experimental group(s) at a specific time point (two-way RM ANOVA and Holm-Sidak post-test). For panels K and L, ψ indicates a significant difference (P<0.05; two-way RM ANOVA and Holm-Sidak post-test) between the heart preparations exposed to graded acidosis from pH 7.8 to 7.0 in anoxia.
without increased adrenaline (black triangles) and with increased adrenaline (black diamonds). Values are means ± SE.

**Fig. 3.** Comparison of the intrinsic contractile properties of 6.5°C spontaneously contracting crucian carp heart preparations exposed to control normoxia ($n = 6$), 40 min of anoxia at pH 7.8, 7.4 or 7.0 and subsequent reoxygenation ($n = 5$). Dissimilar letters indicate significant differences ($P<0.05$) between experimental groups at a specific time point (one-way RM ANOVA and Holm-Sidak post-test). Values are means ± SE.
Fig. 1
Fig. 2
Fig. 3

A: Heart rate (min⁻¹)

B: Maximum contractile force (N g⁻¹)

C: Time-to-peak (s)

D: Time-to-half relaxation (s)

Legend:
- Control Normoxia
- Anoxia at pH 7.8
- Anoxia at pH 7.4
- Anoxia at pH 7.0

Letters indicate significant differences among treatments.