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SHORT COMMUNICATION

The effects of hypoxic bradycardia and extracellular HCO_3^-/CO_2 on hypoxic performance in the eel heart

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ABSTRACT

During hypoxia, fishes exhibit a characteristic hypoxic bradycardia, the functional significance of which remains debated. Here, we investigated the hypothesis that hypoxic bradycardia primarily safeguards cardiac performance. In preparations from the European eel (*Anguilla anguilla*), a decrease in stimulation frequency from 40 to 15 beats min^{-1}, which replicates hypoxic bradycardia *in vivo*, vastly improved cardiac performance during hypoxia *in vitro*. As eels display dramatic shifts in extracellular HCO_3^-/CO_2, we further investigated the effect this has upon hypoxic cardiac performance. Elevations from 10 mmol l^{-1} HCO_3^-/1% CO_2 to 40 mmol l^{-1} HCO_3^-/4% CO_2 had few effects on performance; however, further, but still physiologically relevant, increases to 70 mmol l^{-1} HCO_3^-/7% CO_2 compromised hypoxia tolerance. We revealed a four-way interaction between HCO_3^-/CO_2, contraction frequency, hypoxia and performance over time, whereby the benefit of hypoxic bradycardia was most prolonged at 10 mmol l^{-1} HCO_3^-/1% CO_2. Together, our data suggest that hypoxic bradycardia greatly benefits cardiac performance, but its significance may be context specific.

KEY WORDS: Heart rate, Myocardial hypoxia, Contractility, *Anguilla anguilla*

INTRODUCTION

Fishes exhibit a characteristic slowing of the heart during hypoxia. This enigmatic hypoxic bradycardia is primarily mediated by increased vagal tone and hence reflects a regulated physiological response, but its adaptive significance, albeit extensively debated, remains elusive (Randall, 1982; Perry and Desforges, 2006; Farrell, 2007). Recent studies in eels (Iversen et al., 2010) and other teleosts (Perry and Desforges, 2006; McKenzie et al., 2009) demonstrate that pharmacological or surgical elimination of hypoxic bradycardia does not affect branchial oxygen uptake, suggesting that the potential benefit of the lower heart rate lies elsewhere in the cardiorespiratory system. Intriguingly, it has been suggested that hypoxic bradycardia represents a strategy to protect the heart itself (Farrell, 2007). This hypothesis is partly founded on the negative force–frequency effect of fish hearts, which describes the greater force generation at low heart rates due to the greater potential to reduce diastolic calcium levels and thus increase calcium transients (reviewed in Shiels et al., 2002). This may be particularly relevant during hypoxia and reoxygenation, when myocardial relaxation becomes impaired (e.g. Stecyk et al., 2011; Joyce et al., 2015). By way of analogy, it was recently demonstrated that simulated cold-induced bradycardia 'protects' calcium transients at low temperature in bluefin tuna cardiomyocytes (Shiels et al., 2015).

Eels (*Anguilla* spp.) are particularly tolerant of hypoxia (Wood and Johansen, 1973; Iversen et al., 2010), at least in part because of an exceptional hypoxia tolerance of the heart (Gesser et al., 1982; Davie et al., 1992; Hartmund and Gesser, 1996). Environmental hypoxia is typically associated with other stressors, including hypercapnia, but how such extrinsic factors may modulate myocardial hypoxia tolerance is not well understood. Upon adaptation to freshwater (Farrell and Lutz, 1975) or exposure to chronic hypercapnia (McKenzie et al., 2003), European eels (*A. anguilla*) vastly elevate plasma bicarbonate concentrations ($[HCO_3^-]$) at the expense of chloride (Cl^-). During severe hypercapnia, $[HCO_3^-]$ may exceed 70 mmol l^{-1}, which is unparalleled amongst teleosts and facilitates effective pH regulation (McKenzie et al., 2003). The concomitant rise in HCO_3^-/CO_2 (at constant pH) enhances myocardial hypoxia tolerance in carp (*Cyprinus carpio*), and to a lesser extent rainbow trout (*Oncorhynchus mykiss*) (Gesser, 1977), although we recently failed to report such an effect in the air-breathing fish *Pangasianodon hypophthalmus* (Joyce et al., 2015). It is nevertheless possible that, as in carp, compensatory acid–base changes may confer an additional benefit to the eel heart during hypoxia.

Here, we investigate whether physiologically relevant changes in HCO_3^-/CO_2 and contraction frequency affect hypoxia tolerance in European eel cardiac preparations. We hypothesise that both elevated HCO_3^-/CO_2 and bradycardia confer cardiac protection during oxygen deprivation.

MATERIALS AND METHODS

Experimental animals

European eels (*Anguilla anguilla* [Linnaeus 1758]; 380–1000 g) of undetermined sex were obtained from a local eel farm (Lyksvad fish farm, Denmark) and maintained at Aarhus University in large aquaria (18°C) where they were fed commercial fish food twice a week.

Myocardial preparations

Eels were anaesthetised in an aerated benzocaine solution (0.2 g l^{-1}) until gill ventilation ceased, before the heart was rapidly excised and placed in ice-cold 10 mmol l^{-1} HCO_3^- Ringer's solution (full composition below). Strips of myocardium were prepared and tied at each end with surgical silk, so one end could be secured to a metal rod attached to a force transducer, whilst the other end was anchored to one of two platinum electrodes in an organ bath containing 50 ml of Ringer's solution at 18°C. The two platinum electrodes were connected to Grass stimulators (SD9), which provided 5 ms pulses at 0.66 Hz (40 beats min^{-1}) with a voltage adjusted to twice the minimum required to invoke full contraction. Preparations were then stretched with a micrometer screw to attain maximum force.

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Received 28 August 2015; Accepted 16 November 2015

Journal of Experimental Biology

Journal of Experimental Biology (2016) 219, 302-305 doi:10.1242/jeb.130971

Experimental protocol

Three different Ringer's solutions and gas mixtures were employed. In the first, 10 mmol l^{-1} HCO_3^- Ringer's solution [NaCl (120 mmol l^{-1}), $NaHCO_3$ (10), KCl (4), NaH_2PO_4 (1), CaCl (3.5) and glucose (5)] was bubbled with 1% CO_2, 49% N_2 and 50% O_2. In the two other conditions, to elevate HCO_3^- whilst maintaining Na^+ constant (131 mmol l^{-1}), NaCl was exchanged for equimolar $NaHCO_3$. $NaHCO_3$ was increased to 40 or 70 mmol l^{-1}, whilst NaCl was reduced to 90 or 60 mmol l^{-1}, respectively. In parallel, CO_2 was elevated to 4 and 7% at the expense of N_2, thus maintaining a pH of 7.65. This represents the range of HCO_3^-/CO_2 reported in eels by McKenzie et al. (2003). CaCl was also elevated to 4 and 4.5 mmol l^{-1} to mitigate for the effects of $CaHCO_3$ formation (Schaer, 1974) and further mimic the conditions reported by McKenzie et al. (2003) during hypercapnia.

Four strips were run in parallel at the same HCO_3^-/CO_2 level. After at least an hour of stabilization, two strips were rendered severely hypoxic by exchanging O_2 for N_2 for 30 min. During this period, the stimulation frequency of one normoxic and one hypoxic strip was decreased to 0.25 Hz (15 beats min^{-1}) to resemble the hypoxic bradycardia in eels (Iversen et al., 2010). Preparations were then re-oxygenated for 10 min at 0.66 Hz. The mass and length of the strips were measured upon completion of the experiment so that force could be calculated relative to cross-sectional area (e.g. Gesser, 1977). Preparations were between 5 and 10 mm in length with a mean (±s.e.m.) cross-sectional area of 1.40±0.14 mm^2.

Analyses

To investigate contractile force and diastolic dynamics, twitch force and the rate of 50% relaxation were recorded 10 min before hypoxia and at 5 min intervals thereafter. An additional recording was taken 2.5 min after reoxygenation to increase the temporal resolution during this critical period.

Statistical analysis was carried out in R and Prism (GraphPad). A one-way ANOVA was carried out on the absolute values of twitch force and rate of 50% relaxation at the start of the different experimental protocols. As there were no initial differences in force (P=0.33) or rate of 50% relaxation (P=0.27) between conditions, all values were normalised to those measured 10 min before hypoxia. The effects of time, hypoxia exposure, HCO_3^-/CO_2 and stimulation frequency on relative changes in force and rate of 50% relaxation were analysed using a linear mixed effects model with hypoxia, HCO_3^-/CO_2 and stimulation frequency treated as factors. All values are presented as means±s.e.m.

RESULTS AND DISCUSSION

A representative trace of contractile strength at 10 mmol l^{-1} HCO_3^- and 1% CO_2 is depicted in Fig. 1. The linear mixed effects model revealed that time (P<0.001), hypoxia (P<0.001), stimulation frequency (P<0.001) and HCO_3^-/CO_2 (P=0.003) all exerted independent effects on the relative change in twitch force. Further, hypoxia reduced the rate of relaxation, which persisted during re-oxygenation (P<0.001; Fig. 2).

There was a significant interaction between stimulation frequency and hypoxia (P<0.001; Fig. 2), whereby physiologically relevant decreases in stimulation frequency, to mimic the hypoxic bradycardia measured *in vivo* (Iversen et al., 2010), vastly improved hypoxia tolerance of the eel myocardium. This strongly supports Farrell's (2007) hypothesis that hypoxic bradycardia foremost protects cardiac performance. Indeed, at the frequencies investigated, there appeared to be little force–frequency dependency during normoxia, although this was accentuated at elevated HCO_3^-/CO_2. In other words, severe hypoxia intensified the negative force–frequency effect. The benefit of lower heart rates during hypoxia is reminiscent of that previously characterised in fish during acute and chronic temperature changes (Shiels et al., 2002, 2015).

At low contraction frequencies, cardiomyocytes are better able to reduce diastolic calcium levels and thus increase the activator calcium transient (Harwood et al., 2000; Shiels et al., 2002). As relaxation becomes particularly impaired during hypoxia (Stecyk et al., 2011; Joyce et al., 2015), the greater inter-contraction interval will benefit calcium exudation during diastole. Further, in the present study we revealed greater rates of relaxation during hypoxia

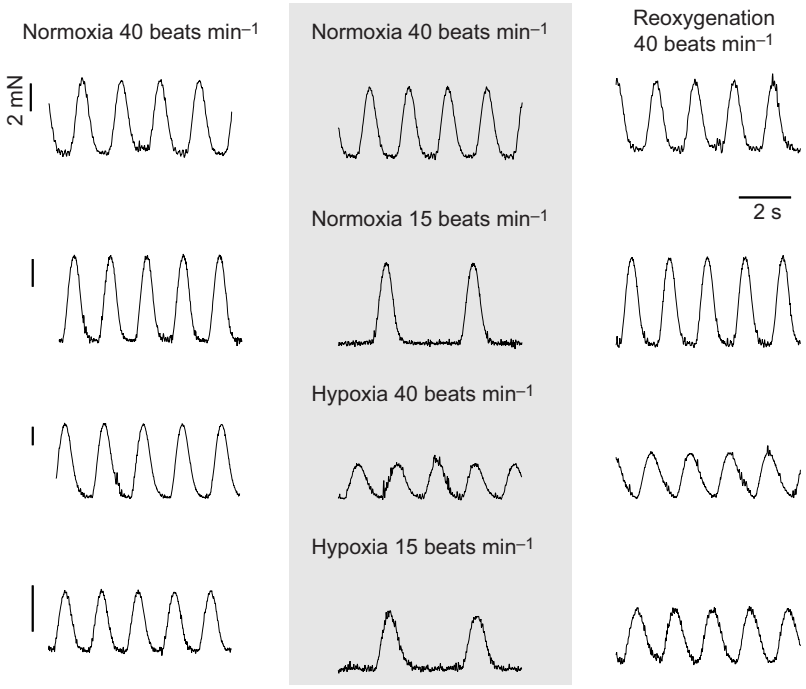

Fig. 1. Original and representative traces depicting the effect of hypoxia (30 min) and reoxygenation (5 min) and changes in stimulation frequency in eel (*Anguilla anguilla*) cardiac preparations (10 mmol l^{-1} HCO_3^-/1% CO_2; pH 7.65).

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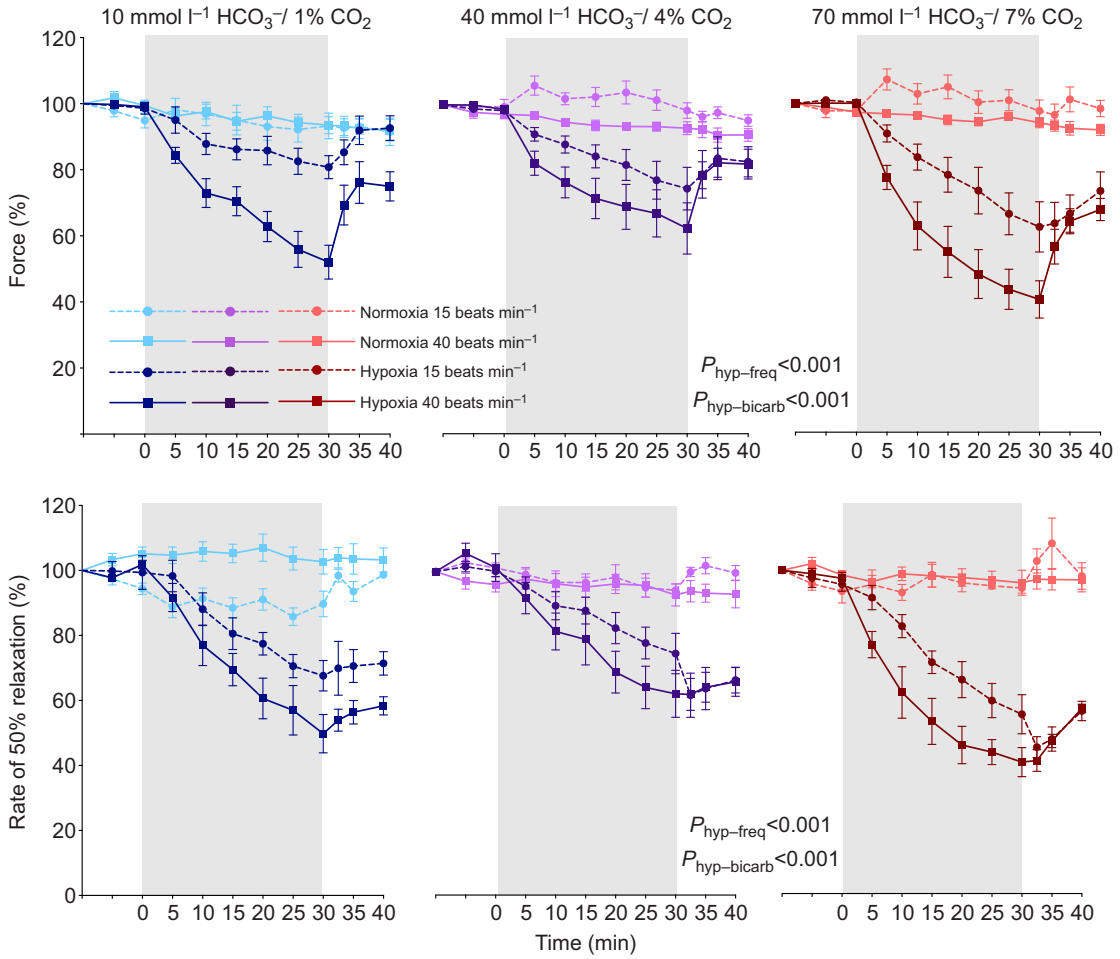

Fig. 2. The effect of stimulation frequency and variable HCO_3^-/CO_2 (pH 7.65) on twitch force and rate of 50% relaxation in eel (*Anguilla anguilla*) cardiac preparations during hypoxia and reoxygenation. The shaded area demarcates when preparations were made hypoxic and/or stimulation frequency was changed (see key). *P*-values represent the interaction between hypoxia (hyp) treatment and stimulation frequency (freq) or HCO_3^-/CO_2 (bicarb) (linear mixed effects model). n=7 in all cases except for 10 mmol l^{-1} HCO_3^-/1% CO_2, normoxia 15 beats min^{-1}, in which case n=6.

at lower stimulation frequencies (Fig. 2). In the *in vivo* situation this is likely to reduce end-diastolic volume and thus maintain large stroke volumes during oxygen deprivation.

The hypoxic bradycardia may also benefit other aspects of cardiac calcium handling and excitation–contraction coupling. For example, low heart rates afford prolonged action potentials (Harwood et al., 2000; Shiels et al., 2015), which may increase the activator calcium influx during hypoxia and compensate for lowered calcium sensitivity by the myofilaments as inorganic phosphates rise (Driedzic and Gesser, 1994; Jensen and Gesser, 1999).

An additional value of hypoxic bradycardia may reside in myocardial energetics (Farrell, 2007). By estimating cardiac work from the product of contraction frequency and force, our data reveal that bradycardia may reduce myocardial energy demand by up to 50%, despite the increased twitch force. Indeed, because fish hearts display a characteristic ability to increase stroke volume at low heart rates (‘intrinsic autoregulation of cardiac output’ *sensu* Altimiras and Axelsson, 2004), the hypoxic bradycardia does not necessarily decrease cardiac output, although cardiac output of eels does decrease in very severe hypoxia (Iversen et al., 2010). ‘Saving’ energy on excitation–contraction coupling has the dual benefit of conserving cardiac energy stores and minimising the accumulation of lactic acid. Further, the prolonged diastolic period may favour the removal of inorganic phosphates by glycolysis and prolong the time for energy restoration at ATPase sites.

Because eels exhibit large *in vivo* elevations in [HCO_3^-] to above 70 mmol l^{-1} (McKenzie et al., 2003), i.e. more than twice that investigated in other species (Gesser, 1977; Joyce et al., 2015), we reasoned that any potential benefit of elevated HCO_3^-/CO_2 would be maximally revealed in the eel heart. However, in contrast to our initial hypothesis, increasing HCO_3^-/CO_2 to 70 mmol l^{-1} and 7%, respectively, reduced both force generation and the rate of relaxation during hypoxia (P<0.001; Fig. 2). Preparations at high HCO_3^-/CO_2 also performed particularly poorly during re-oxygenation.

When comparing with other fish species, such as the common carp in which protective effects of elevated HCO_3^-/CO_2 appear most marked (Gesser, 1977), it is important to note that eel preparations were considerably more hypoxia tolerant than carp (20% reduction in force versus 65% reduction in force during similar experimental conditions) at similar and low HCO_3^-/CO_2 concentrations (10 mmol l^{-1}/1% and 6.25 mmol l^{-1}/1%, respectively). However, at higher HCO_3^-/CO_2 (40 mmol l^{-1}/4% and 25 mmol l^{-1}/4%), eels and carp compared much more favourably (20–25% reduction in force in both cases). This suggests that eel myocardium cannot benefit from the additional

increases in bicarbonate that the carp heart can exploit. Indeed, it is not surprising that the hypoxia tolerance of the eel myocardium could not be improved given that, at low heart rate, hypoxic preparations were remarkably similar to normoxic controls. The detrimental effect of 70 mmol l^{-1} HCO_3^-/7% CO_2 was unexpected, but may be rationalised based on the acute nature of the experiment. It is possible that the preparations may have been compromised by the low [Cl^-] needed to compensate for elevated [HCO_3^-]. In the mammalian heart, chloride currents appear important in the recovery from ischaemia–reperfusion events (Petrich et al., 1996; Uramoto et al., 2012), but this remains to be investigated in piscine hearts. Given that elevated plasma [HCO_3^-] levels in response to severe hypercapnia did not affect *in vivo* hypoxia tolerance in eels (McKenzie et al., 2003), the potential detriment reported in our study may be compensated for during acclimation.

HCO_3^-/CO_2 affected the importance of stimulation frequency during hypoxia over time (i.e. there was a four-way interaction between time, HCO_3^-/CO_2, hypoxia treatment and stimulation frequency, $P<0.001$). The beneficial effect of bradycardia was clearly discernible within the first 10 min of hypoxia, whilst twitch force declined linearly thereafter. To further explore this relationship, linear regressions were performed on the fall of twitch force between 10 and 30 min of hypoxia exposure at different HCO_3^-/CO_2 concentrations. At elevated HCO_3^-/CO_2 (40 or 70 mmol l^{-1} HCO_3^- and 4 or 7% CO_2), the slopes of 15 and 40 beats min^{-1} preparations were indistinguishable ($P=0.95$ in both cases). However, at 10 mmol l^{-1} HCO_3^- and 1% CO_2, preparations paced at 40 beats min^{-1} had a significantly steeper slope than those at 15 beats min^{-1} ($P=0.04$). Thus, lower HCO_3^-/CO_2 prolonged the benefit of hypoxic bradycardia. This difference was further evident during reoxygenation, when only bradycardic 10 mmol l^{-1} HCO_3^-/1% CO_2 preparations fully recovered twitch force. The preparations that did not fully recover force may have incurred permanent damage during the hypoxic insult. Further, at elevated HCO_3^-/CO_2, the rate of relaxation during reoxygenation in preparations stimulated at different frequencies entirely converged, whereas at 10 mmol l^{-1} HCO_3^- and 1% CO_2, bradycardic preparations maintained faster relaxation rates during recovery.

In conclusion, our data support the notion that the hypoxic bradycardia benefits myocardial performance, but also emphasise that the functional importance of this benefit depends on other environmental and physiological factors.

Acknowledgements
We thank Dr Anthony (Tony) Farrell for constructive discussions at the Society for Experimental Biology annual main meeting. We are also indebted to Dr Heath MacMillan for statistical advice.

Competing interests
The authors declare no competing or financial interests.

Author contributions
W.J. conceived and designed the experiments, M.S. and W.J. performed the experiments and analysed the data, and all authors contributed to writing the paper.

Funding
This study was funded by the Danish Research Council.

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