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Differential effects of bicarbonate on severe hypoxia- and hypercapnia-induced cardiac malfunctions in diverse fish species

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Abstract

We tested in six fish species [Pacific lamprey (*Lampetra richardsoni*), Pacific spiny dogfish (*Squalus suckleyi*), Asian swamp eel (*Monopterus albus*), white sturgeon (*Acipenser transmontanus*), zebrafish (*Danio rerio*), and starry flounder (*Platichthys stellatus*)] the hypothesis that elevated extracellular [HCO_3^-] protects spontaneous heart rate and cardiac force development from the known impairments that severe hypoxia and hypercapnic acidosis can induce. Hearts were exposed *in vitro* to either severe hypoxia (~3% of air saturation), or severe hypercapnic acidosis (either 7.5% CO_2 or 15% CO_2), which reduced heart rate (in 6 test species) and net force development (in 3 test species). During hypoxia, heart rate was restored by [HCO_3^-] in a dose-dependent fashion in lamprey, dogfish and eel (EC_{50} = 5, 25 and 30 mM, respectively), but not in sturgeon, zebrafish or flounder. During hypercapnia, elevated [HCO_3^-] completely restored heart rate in dogfish, eel and sturgeon (EC_{50} = 5, 25 and 30 mM, respectively), had a partial effect in lamprey and zebrafish, and had no effect in flounder. Elevated [HCO_3^-], however, had no significant effect on net force of electrically paced ventricular strips from dogfish, eel and flounder during hypoxia and hypercapnia. Only in lamprey hearts did a specific soluble adenylyl cyclase (sAC) inhibitor, KH7, block the HCO_3^--mediated rescue of heart rate during both hypoxia and hypercapnia, the only species where we conclusively demonstrated sAC activity was involved in the protective effects of HCO_3^- on cardiac function. Our results suggest a common HCO_3^--dependent, sAC-dependent transduction pathway for heart rate recovery exists in cyclostomes and a HCO_3^--dependent, sAC-independent pathway exists in other fish species.

Keywords

Bicarbonate ions; Cardiac contractility; Heart rate; Hypercapnia tolerance; Hypoxia tolerance; Soluble adenylyl cyclase

Introduction

Severe hypoxia and hypercapnic acidosis, which can exist in nature, are known to severely impair heart rate and cardiac force development in a wide variety of fish species both *in vivo* and *in vitro*. Species variability is evident, however, for the debilitating effects of severe hypoxia on the heart rate and cardiac force development of fishes. For example, just 6 min of hypoxic exposure in Atlantic cod (*Gadus morhua*) reduced *in vivo* heart rate (Fritsche and Nilsson 1988), while isolated hearts from rainbow trout (*Salmo gairdneri* now *Oncorhynchus mykiss*) experienced a 70% reduction in spontaneous heart rate after 1 h of progressive hypoxia (Marvin and Burton 1973). Debilitating effects of hypoxia were also reported for isolated cardiac strips, which reduced force development by ~50% after only a 10 min exposure to anoxia for Atlantic cod (Gesser and Poupa 1983), and after 30 min in plaice (*Pleuronectus platessa*). In contrast, hypoxic bradycardia was weak or absent in winter flounder (*Pseudopleuronectes americanus*) (Cech et al. 1977) and in two species of armored catfishes (*Pterygoplichthys gibbiceps* and *Liposarcus pardalis*) (MacCormack et al. 2003). Also, hypoxia did not affect spontaneous heart rate in isolated hearts from sea raven (*Hemitripterus americanus*) (Farrell et al. 1985).

Similarly, studies on the effects of severe hypercapnia on heart rate and cardiac force development have also revealed species variability. Bradycardia was a common, albeit species-specific response of fishes to hypercapnia (Perry and Gilmour 2002). For example, while

exposure to ~1% CO_2 reduced *in vivo* heart rate by between ~20% and 70% in Pacific spiny dogfish (*Squalus acanthias*, now *S. suckleyi*), Pacific sanddab (*Citharychthus sordidus*) and salmonids, heart rate was unchanged in American eel (*Anguilla ro`strata*) or brown bullhead catfish (*Ameiurus nebulosus*) (Perry and Gilmour 2002). With ~2.6% CO_2, heart rate increased by 8% in white sturgeon (*Acipenser transmontanus*) (Crocker et al. 2000). Conversely, exposure to a higher CO_2 concentration (5%) consistently reduced *in vivo* heart rate by between 25% and 50% in tiger fish (*Hoplias malabaricus*) (Reid et al. 2000), tambaqui (*Colossoma macropomum*) (Sundin et al. 2000) and dogfish (*S. acanthias*) (Kent and Peirce 1978). The debilitating effects of severe hypercapnia (>7.5% CO_2) on cardiac force development have been extensively examined in isolated heart strips and again species-specific differences have emerged. Hypercapnic acidosis decreased force development by >50% after 30 min of exposure in many species, including rainbow trout, carp (*Cyprinus carpio*), Atlantic cod and the air-breathing mud eel (*Synbranchus marmoratus*) (Gesser and Poupa 1979; Hansen and Gesser 1980; Gesser and Jorgensen 1982; Gesser and Poupa 1983). A biphasic response to hypercapnic acidosis, however, was reported for European flounder (*Pleuronectus nesus*), plaice (Gesser and Poupa 1979; Gesser and Jorgensen 1982) and armored catfish (*Pangasianodon hypopthalamus*) (Joyce et al. 2015), whereby an initial and sizeable decrease in force development was followed by spontaneous recovery to control levels.

While some of the variability seen in these responses could be truly attributed to species differences, some of the variability likely reflected differences in the severity of the challenge. Also, different results seen for *in vivo* and *in vitro* situations may reflect cardio-protective mechanisms being expressed *in vivo*. An established cardio-protective mechanism is related to the availability of extracellular bicarbonate (HCO_3^-) because hypoxia and hypercapnia-induced

cardiac malfunction in fishes can be somehow ameliorated in a dose-dependent manner by increasing the extracellular [HCO_3^-] (Poupa and Johansen 1975; Gesser and Poupa 1979; Gesser and Jorgensen 1982; Gesser and Poupa 1983). Yet again, species variability exists for the protective role of extracellular [HCO_3^-]. For example, although elevated external [HCO_3^-] partially rescued cardiac function in Atlantic cod, a complete recovery that surpassed control levels at the highest test [HCO_3^-] (~40 mM) was possible in flatfishes (flounder and plaice). Thus, while the protective effect of HCO_3^- seems to be more pronounced in those species that show a cardiac biphasic response to extreme hypercapnia, the heterogeneity of cardiac preparations and saline compositions used in the various studies limits any attempt to rigorously synthetize these previous results and hypothesize about unifying mechanisms.

Our focus on the direct cardiac effects of extracellular HCO_3^- during severe hypoxia and hypercapnia reflected, in part, the recent discovery of a novel, HCO_3^--dependent mechanism that modulates heart rate in the Pacific hagfish (*Eptatretus stoutii*) (Wilson et al. 2016). This mechanism depends on soluble adenylyl cyclase (sAC), an enzyme directly stimulated by HCO_3^- to produce the messenger molecule cyclic adenosine monophosphate (cAMP) (Chen et al. 2000). The stimulatory mechanism involves the binding of HCO_3^- to specific amino acid residues in the catalytic site, which favors the binding of substrate ATP and its cyclization into cAMP (Steegborn et al. 2005; Kleinboelting et al. 2014). Unlike the traditional transmembrane adenylyl cyclases (tmACs), sAC is not regulated by G protein-coupled receptors (GPCRs) and it does not have any transmembrane domains. Instead, sAC can be found in various intracellular compartments including the cytoplasm and the nucleus (reviewed in Tresguerres et al. 2011). Together with phosphodiesterases that degrade cAMP and restrict its diffusion, the presence of multiple cAMP sources within a cell has led to the "cAMP signaling microdomain" model

(Zaccolo 2009). Briefly, this model implies that cAMP acts on target effector proteins in rather defined subcellular compartments, and could explain how the same signaling molecule, cAMP, might produce diverse and sometimes opposite effects on cell physiology (reviewed in Tresguerres et al. 2019).

Previously, sAC-mediated control of heart rate has been examined in isolated, spontaneously beating hagfish hearts after exposure to severe hypoxia, when the normoxic heart rate had been halved and the catecholamine-mediated branch of the cAMP signaling cascade that normally acts through GPCR β-adrenoceptors and tmACs was ineffective (Nilsson 1983; Wilson et al. 2016). Specifically, addition of HCO_3^- to severely hypoxic isolated hagfish hearts induced a dose-dependent increase of spontaneous heart rate, surpassing the normoxic heart rate by ~75% in the presence of 40 mM HCO_3^-. Furthermore, this recovery of heart rate during severe hypoxia with elevated [HCO_3^-] conditions was blocked by the small molecule KH7, a specific sAC inhibitor (Tresguerres et al. 2010; Bitterman 2013). Although sAC also is present in the hearts of the leopard shark (*Triakis semifasciata*) (Roa and Tresguerres 2017) and rainbow trout (Salmerón et al. in press), its putative role in modulating heart rate and perhaps force development in fish species other than in hypoxic hagfish hearts has not been investigated.

Consequently, the main objective of the current study was to determine if the reported protective effects of HCO_3^- on cardiac function under severe hypoxia and hypercapnia, especially those mediated through sAC, apply more broadly to fishes. Therefore, we surveyed cardiac function in six fish species using the same techniques and standardized treatment conditions in the bath solutions. The six species were: (1) the Pacific lamprey (*Lampetra richardsoni*; a locally available cyclostome and sister group to the hagfishes); (2) the Pacific spiny dogfish (*S. suckleyi*; a locally available elasmobranch); (3) the white sturgeon (*Acipenser*

transmontanus; a locally available non-teleost bony fish known for its hypoxia and hypercapnia tolerance); (4) the starry flounder (*Platichthys stellatus*) (a locally available bony fish that is closely related to the flatfish species used in some of the previous studies mentioned above); (5) the Asian swamp eel (*Monopterus albus*; a locally available air-breathing bony fish); and (6) the zebrafish (*Danio rerio*; a locally available freshwater bony fish gaining prominence as a model species). For all species, we examined the effect of cumulative addition of HCO_3^- on the spontaneous heart rate of isolated hearts exposed to severe hypoxia or hypercapnia. We controlled for the alkalinizing effect of adding HCO_3^- on pH by examining in parallel experiments the effect of an equivalent pH change using NaOH addition. We additionally examined net force development of isolated ventricular strips in the three larger fish species (i.e. dogfish, flounder and swamp eel). The potential role of sAC in HCO_3^--mediated cardiac protection was tested by adding KH7 to block any sAC involvement due to HCO_3^- additions. Under our experimental conditions, sAC played a role only in the lamprey heart, leading to an initial immunohistochemical examination that provided further evidence for the presence of sAC in the lamprey heart.

Materials and Methods

Animal husbandry

All experiments were approved by the Animal Care Committee of the University of British Columbia (A16-0038) and the Centre for Aquaculture and Environmental Research (16-0038-001A2) and conducted in accordance with the Canadian Council on Animal Care guidelines. The representative fish species were all locally available either as wild-captured or commercially purchased. They included species that live in or on sediments (juvenile lampreys,

white sturgeon, starry flounder and Asian swamp eels) where hypoxic and hypercapnic conditions likely prevail. Juvenile lampreys (0.31 ± 0.02 g; mean ± s.e.m.) were collected using dip nets from the Fraser River near Langley, British Columbia. The collected specimens were likely mixed species (*Lampetra richardsoni* and *Entosphenus tridentatus*) as both species are present at the collection site and juveniles of these two species cannot be visually distinguished from each other (personal communication, Dr. R. Beamish). Lampreys were transported to the University of British Columbia (UBC) and held in 40 L glass tanks with recirculating and filtered freshwater at 10 °C, where they were fed baker's yeast once per week. Spiny dogfish (*S. suckleyi*; 1.48 ± 0.06 kg) were caught using rod and reel in English Bay near the Centre for Aquaculture and Environmental Research, West Vancouver, British Columbia where they were held in 2,000 L fiberglass tanks with flow-through, aerated seawater at 9-11 °C and fed frozen squid and salmon until satiation three times per week. Juvenile white sturgeon (*A. transmontanus*; 14.4 ± 0.7 g) transported from the Vanderhoof Hatchery in Vanderhoof, British Columbia to UBC and held in 400 L fiberglass tanks with recirculating, filtered freshwater at 10 °C. Sturgeon were fed trout pellets until satiation three times a week. Starry flounder (*P. stellatus*; 57.3 ± 4.1 g) also were caught in English Bay near West Vancouver but using seine nets. After transport to UBC they were kept in recirculating, filtered 400 L fiberglass tanks containing aerated artificial saltwater (Instant Ocean Salt Mix, Aquarium Systems, Mentor, OH) at 30 ppt at 10 °C and were fed with shrimp until satiation three times a week. Asian swamp eels (*M. albus*; 120.5 ± 10.4 g) were purchased live from a local seafood market (Parker Seafood, Richmond, British Columbia). At UBC they were held in 400 L fiberglass tanks with recirculating, filtered freshwater at 15 °C and were fed with shrimp until satiation three times a week. Zebrafish (*D. rerio*; 0.32 ± 0.03 g) were purchased from a local store (Petsmart,

Vancouver, British Columbia) and at UBC they held in 40 L glass tanks with recirculating, filtered freshwater at 20 °C. Zebrafish were fed with commercial staple flake food until satiation three times a week.

Saline composition, pH and [HCO_3^-] measurements for the in vitro heart preparations

The physiological saline reflected the blood plasma composition of each species. A freshwater fish saline was used for lamprey, sturgeon, swamp eel, and zebrafish hearts and consisted of (in mM): 125 NaCl, 2.5 KCl, 0.9 $MgSO_4$, 2.5 $CaCl_2$, 5.6 glucose, 3.9 TES free acid, and 6.1 TES Na^+ salt in distilled H_2O (modified from Farrell et al. 1996). A saltwater fish saline was used for flounder hearts and had the same composition except with 180 mM NaCl (Gesser and Poupa 1979). The saline used with dogfish consisted of (in mM): 260 NaCl, 5 KCl, 3 $CaCl_2$, 1.33 $MgSO_4$, 5.6 glucose, 1 Na_2HPO_4, 350 urea, and 70 TMAO (modified from Dombkowski et al. 2004). All control salines were adjusted to pH 7.8 with 1 M NaOH (Mettler Toledo SevenEasy pH meter with an attached Mettler Toledo InLab 413 SG probe, Schwerzenbach, Switzerland). HCO_3^- was added using a 1 M $NaHCO_3$ stock. To determine experimental conditions in the saline (Table 1), each treatment was repeated three times where pH and [HCO_3^-] were then measured. P_{CO2} of the saline (calculated using % CO_2 * 760 mmHg) and total CO_2 (measured using a Corning 965 Carbon Dioxide Analyser, Corning Ltd., Halstead, England) were used to calculate [HCO_3^-] (total $CO_2 = \alpha P_{CO2} + [HCO_3^-]$) and the solubility coefficient (α) was calculated using $\alpha = 0.0307 + 0.00057\ (37\ ºC - \text{temp}) + 0.00002\ (37\ ºC - \text{temp})^2$ (Kelman 1967). Bubbling saline with hypercapnic gases increases the [HCO_3^-] somewhat, and so we report the nominal [HCO_3^-].

208 *Stock solutions*

209 Stock solutions of 100 mM KH7 (Tocris Bioscience, Minneapolis, MN, USA) were
210 prepared in DMSO. This stock was then diluted in saline to reach the desired final concentrations
211 before each experiment.

212

213 *Heart isolation*

214 After fish were euthanized by a swift blow to the head, followed by immediate pithing of
215 the brain, the whole heart was quickly excised into appropriate physiological saline at the
216 experimental temperature (Table 1) and bubbled with 100% O_2 to minimize regional hypoxia
217 during tissue preparation. Experiments used the whole heart for lamprey, sturgeon, swamp eel
218 and zebrafish. However, limited numbers of dogfish and flounder required that half of the
219 ventricle was used for isometric force measurements, leaving the atrium and sinus venosus
220 undisturbed (spontaneous atrial beating rates remained statistically unchanged 30 min after
221 halving the ventricle). Each heart was only exposed to either a hypoxia treatment or a
222 hypercapnic acidosis treatment, not both.

223

224 *Heart rate measurements*

225 The rate of the spontaneous heartbeat was measured in a dish with 20 mL of the
226 appropriate saline at the experimental temperature (Table 1), maintained by placing the dish in a
227 water jacket connected to a programmable laboratory chiller (1160S, VWR International, USA).
228 Heart rate was determined from number of atrial beats visually counted over a 1 min period
229 using a dissecting microscope. Atrial beating settled to a steady rate during the initial 30 min,
230 which was normalized to 100% as the pre-exposure control heart rate.

The saline bath was rendered severely hypoxic by bubbling with 100% N_2. Complete anoxia was not attained because bubbling agitated the saline surface, introducing some O_2 back into the saline (monitoring with a fibre optic oxygen probe (Firesting, Pyroscience, Germany) revealed that oxygen in the saline was around 3% air saturation and as low as 1% but never greater than 5%). Hypercapnic acidosis exposure was achieved by similarly bubbling the saline bath with a gas mixture (either 7.5% CO_2 : 92.5% O_2, or 15% CO_2 : 85% O_2) from a gas mixing pump (Wosthoff, Bochum, West Germany), again until heart rate decreased to a stable level. The 15% CO_2 level for hypercapnic acidosis was used for dogfish, flounder and swamp eel to facilitate comparisons with previous studies (Gesser and Poupa 1979; Gesser and Jorgensen 1982; Gesser and Poupa 1983; Yee and Jackson, 1984; Salas et al. 2006; Joyce et al. 2015). However, 7.5% CO_2 was used for hearts from lamprey, sturgeon and zebrafish because they stopped beating within 30 min of exposure to 15% CO_2. Once the decrease in heart rate (expressed as a percent decrease from the normoxic heart rate) reached a stable level (from 30 min to 2 h), hypoxic and hypercapnic hearts were treated with one of four different protocols. Two treatments tested the dose-response to $NaHCO_3$ additions that were added every 15 min to increase the [HCO_3^-] in 10 mM increments up to a final concentration of 50 mM (40 mM for lamprey). At that time, one treatment group received KH7 (final concentration 50 μM in DMSO), and the other treatment group received an equivalent volume of DMSO as a carrier control. The third treatment group, similarly added NaOH incrementally to the saline bath every 15 min as a control group for the extracellular pH and [Na^+] changes associated with the $NaHCO_3$ additions. The $NaHCO_3$ and NaOH stocks were pre-equilibrated with 100% N_2 for the severe hypoxia experiments. The fourth treatment group tested for spontaneous recovery of heart

253 rate during either hypoxia or hypercapnic acidosis. In this group, the preparation was left
254 undisturbed in the debilitating condition for the same duration as the other three treatments.
255
256 *Maximum isometric force measurements*
257 Maximum isometric force generation was measured in ventricular strips (<1 mm width,
258 ~4 mm long), but only for the three larger test species (dogfish, swamp eel and flounder),
259 following the methods detailed in Shiels and Farrell (1997). Briefly, a myocardial strip was
260 secured between a fixed stainless-steel post and an isometric force transducer (MLT0202,
261 ADInstruments, Sydney, Australia) with surgical silk and immersed in one of four water-
262 jacketed organ baths containing 20 mL of the appropriate saline that were bubbled with 100% O_2
263 at the experimental temperature (Table 1). Four preparations from the same heart allowed the
264 four treatment groups to be studied simultaneously on one fish. Preparations were equilibrated
265 for 10 min before electrical stimulation started at 0.2 Hz (two silver electrode plates on either
266 side of the muscle strip delivering 5 V, 10 ms pulses from a Grass SD9 stimulator, Quincy,
267 Massachusetts). Preparations were then stretched with a micrometer screw to reach their
268 maximum isometric force and were left to stabilize for 1 h. Ventricular strips then were exposed
269 to either hypoxia or hypercapnic acidosis and one of the same four treatments, as described
270 above. Signals from the four transducers were recorded and analyzed with data acquisition
271 software (AcqKnowledge, Biopac Systems, Goleta, California). Net force of contraction was
272 calculated using the difference between minimum and maximum tension over 30 s. Net force of
273 contraction was then expressed as mN mm^{-2} using the cross-sectional area of the myocardial
274 strips (estimated at the end of the experiment by measuring length with digital calipers and wet
275 mass, assuming a uniform thickness and a density of 1.06 g/cm^3; Layland et al. 1995).

276

277 *Immunostaining*

278 Excised lamprey hearts were immediately rinsed in ice cold saline and immersed in
279 fixative (3% paraformaldehyde, 0.35% glutaraldehyde in 0.1 M Sodium Cacodylate, pH 7.4,
280 Electron Microscopy Sciences, Hatfield, PA, USA) at 4 ºC for 5 h. Hearts were then transferred
281 to 50% ethanol for 5 h, and finally to 70% ethanol for storage at 4 ºC. Hearts were
282 immunostained and imaged as described in Wilson et al. (2016). Briefly, 7 μm paraffin
283 histological sections were mounted onto glass slides, incubated in blocking buffer (PBS, 2%
284 normal goat serum, 0.02% keyhole limpet hemocyanin, pH 7.8) for 1 h, and then in anti-dogfish
285 shark sAC antibodies (12 μg/ml) (Tresguerres et al. 2010) overnight at 4 °C. Slides were washed
286 three times in PBS and sections were incubated in the secondary antibody (goat anti-rabbit Alexa
287 Fluor 488; Invitrogen, Grand Island, NY; 1:500) at room temperature for 1 h, followed by
288 incubation with the nuclear stain Hoechst 33342 (Invitrogen, Grand Island, NY, USA; 5 μg/ml)
289 for 5 min. Slides were then washed three times in PBS and mounted in Fluorogel with Tris buffer
290 (Electron Microscopy Sciences). Sections incubated without primary antibody, or with antibody
291 pre-incubated with 3X excess antigen peptide ("peptide pre-absorption") served as controls.
292 Immunofluorescence was visualized using an epifluorescence microscope (Zeiss AxioObserver
293 Z1) connected to a metal halide lamp and with the appropriate filters. Digital images were
294 adjusted for brightness and contrast only using Zeiss Axiovision software.

295

296 *Statistical analysis*

297 Data were graphically displayed as percent of pre-treatment value prior to exposure to
298 hypoxia and hypercapnic acidosis to allow for direct comparisons among the test species.

However, statistical differences among values for control and treatment groups were tested using absolute values and a one-way repeated measures ANOVA followed by Holm-Sidak *post-hoc* test. Comparisons between sets of treatments were tested using one-way ANOVA followed by Holm-Sidak test. Data were transformed before analysis if they did not meet assumptions of normality (Kolmogorov-Smirnov test) and equal variance (Levene Median test). Statistical significance was assessed as $P < 0.05$. All statistical analysis was performed using SigmaPlot 12.0 (Systat Software Inc.; www.sigmaplot.com). Figures were produced using GraphPad Prism 6.0 (San Diego, CA, USA). Values are presented as mean ± s.e.m unless otherwise stated.

Results

Effects of HCO_3^- on heart rate during severe hypoxia

Exposure to severe hypoxia significantly decreased spontaneous heart rate in all six species tested (Fig. 1). The decrease in heart rate was ~32% in lamprey (from 37.3 ± 1.0 min^{-1} to 25.5 ± 1.2 min^{-1}) (Fig. 1A), ~34% in dogfish (from 20.5 ± 0.9 min^{-1} to 13.5 ± 0.6 min^{-1}) (Fig. 1B), ~27% in swamp eel (from 38.0 ± 2.2 min^{-1} to 27.5 ± 1.6 min^{-1}) (Fig. 1C), ~43% in sturgeon (from 29.0 ± 1.0 min^{-1} to 16.5 ± 1.5 min^{-1}) (Fig. 1D), ~23% in flounder (from 64.7 ± 4.1 min^{-1} to 49.7 ± 3.2 min^{-1}) (Fig. 1E), and ~44% in zebrafish (from 113.7 ± 9.8 min^{-1} to 64.0 ± 9.7 min^{-1}) (Fig. 1F).

DMSO, the carrier for KH7, had no effect on lamprey heart rate (Fig. 1). The effects of adding $NaHCO_3$, NaOH, and the sAC inhibitor KH7 under severe hypoxic conditions on heart rate were species-specific. In lamprey hearts (Fig. 1A), the control normoxic heart rate was restored by 10 mM HCO_3^- [half-maximal stimulation (EC_{50}) ~ 5 mM]. The hypoxic lamprey heart rate was further stimulated to 43.7 ± 3.8 min^{-1} with 30 mM HCO_3^- despite the continuous

hypoxic exposure, a rate 17% higher than that of normoxic hearts. Addition of NaOH had no significant effect (Fig. 1A), indicating that increases in external pH and [Na^+] were not involved in the rescue of the lamprey hypoxic heart rate. On the other hand, KH7 reduced the maximally HCO_3^--stimulated heart rate back down to the hypoxic heart rate (23.7 ± 2.6 min^{-1}) (Fig. 1A), implying the HCO_3^--induced protection of heart rate in the severely hypoxic lamprey heart was mediated via sAC.

In dogfish and swamp eel hearts, cumulative additions of $NaHCO_3$ also increased the hypoxic heart rate in a dose-dependent fashion (Fig. 1B, C). These two species, however, were less responsive than the lamprey heart, requiring 40 mM HCO_3^- to restore the normoxic heart rate (dogfish shark EC_{50} ~ 25 mM; swamp eel EC_{50} ~ 30 mM). Again, NaOH additions had no significant effect on the hypoxic heart rate of dogfish and swamp eel (Fig. 1B, C). KH7, however, did not have any effect on the HCO_3^--rescued heart rate during severe hypoxia (Fig. 1B, C), implying a HCO_3^--dependent but sAC-independent protection of heart rate in the dogfish and swamp eel hearts. For sturgeon, flounder and zebrafish hearts, the hypoxic heart rate was unchanged by additions of $NaHCO_3$, NaOH, and KH7 (Fig. 1D, E, F).

Effects of HCO_3^- on heart rate during severe hypercapnic acidosis

Hypercapnic acidosis significantly decreased heart rate in all six species (but to varying degrees) (Fig. 2). The decrease in heart rate was ~81% in lamprey (from 27.0 ± 1.9 min^{-1} to 5.0 ± 0.9 min^{-1}) (Fig. 2A), ~25% in dogfish (from 20.8 ± 0.5 min^{-1} to 15.7 ± 0.9 min^{-1}) (Fig. 2B), ~42% in swamp eel (from 29.5 ± 0.7 min^{-1} to 17.2 ± 1.5 min^{-1}) (Fig. 2C), ~38% in sturgeon (from 30.3 ± 2.4 min^{-1} to 18.7 ± 0.9 min^{-1}) (Fig. 2D), ~39% in flounder (from 49.5 ± 3.7 min^{-1} to 30.3 ± 4.2 min^{-1}) (Fig. 2E), and ~33% in zebrafish hearts (from 101.1 ± 2.9 min^{-1} to 67.2 ± 5.3

345 min^{-1}) (Fig. 2F). No spontaneous recovery of heart rate was observed during severe hypercapnic
346 acidosis for any species. DMSO, the carrier for KH7, had no effect on lamprey heart rate (Fig.
347 2).

348 The effects of adding $NaHCO_3$, NaOH, and the sAC inhibitor KH7 on heart rate during
349 hypercapnic acidosis were species-specific (Fig. 2). Addition of $NaHCO_3$ produced a significant,
350 dose-dependent increase in the hypercapnic heart rate in five species, the exception being
351 flounder where no effect was seen (Fig. 2E). In lamprey, 40 mM HCO_3^- partially rescued the
352 hypercapnic heart rate (from 5.0 ± 0.9 min^{-1} to 16.0 ± 1.3 min^{-1}) and the increase in heart rate
353 was significantly inhibited by KH7 (9.2 ± 0.9 min^{-1}) (Fig. 5A). The HCO_3^--induced recovery of
354 the hypercapnic lamprey heart rate was independent of external pH because NaOH additions had
355 no effect.

356 In dogfish, swamp eel and sturgeon, the hypercapnic heart rate was fully rescued by 50
357 mM HCO_3^-, but KH7 had no significant effect on the recovered heart rate (Fig. 2B-D). Instead,
358 the addition of NaOH in dogfish and sturgeon mimicked the HCO_3^--mediated rescue of
359 hypercapnic heart rate (Fig. 2B, D), suggesting a pH-mediated protection of the hypercapnic
360 heart rate in these two species. In swamp eel, neither NaOH nor KH7 had an effect on the
361 hypercapnic heart rate (Fig. 2C). In zebrafish, cumulative addition of HCO_3^- up to 30 mM
362 progressively increased the hypercapnic heart rate (Fig. 2F). This partial rescue of the
363 hypercapnic heart rate was insensitive to both NaOH and KH7. The zebrafish heart then stopped
364 beating with a higher [HCO_3^-]

365

366 *Effects of HCO_3^- on ventricular isometric force development during severe hypoxia*

Severe hypoxia significantly decreased net force in ventricular strips for the three species tested, the dogfish, swamp eel and flounder (Fig. 3A, B, C). Net force decreased by ~63% in dogfish (from 14.8 ± 0.8 mN mm^{-2} to 5.5 ± 0.6 mN mm^{-2}), by ~70% in swamp eel (from 10.2 ± 0.6 mN mm^{-2} to 3.0 ± 0.5 mN mm^{-2}) and by ~43% in flounder (from 11.3 ± 0.9 mN mm^{-2} to 6.4 ± 0.4 mN mm^{-2}). Addition of $NaHCO_3$ (up to 50 mM) had no effect on hypoxic cardiac net force in dogfish (Fig. 3A), slightly increased hypoxic net force in the swamp eel (up to 4.8 ± 0.6 mN mm^{-2}) (Fig. 3B), and progressively decreased hypoxic net force in the flounder (down to 4.1 ± 0.5 mN mm^{-2}) (Fig. 3C), a decline likely due to progressive deterioration of the preparation rather than being a response to HCO_3^-. Neither NaOH nor KH7 had a significant effect on net force in the three species tested (Fig. 4).

Effects of HCO_3^- on ventricular isometric force development during severe hypercapnic acidosis

Hypercapnic acidosis induced by bubbling with 15% CO_2 significantly decreased net force to varying extents in ventricular strips from the dogfish, swamp eel and flounder (Fig. 3D, E, F). Net force decreased by ~68% in dogfish (from 14.9 ± 1.4 mN mm^{-2} to 4.7 ± 0.8 mN mm^{-2}) (Fig. 3D), by ~35% in swamp eel (from 11.9 ± 0.9 mN mm^{-2} to 7.8 ± 0.5 mN mm^{-2}) (Fig. 3E) and by ~42% in flounder (from 10.7 ± 0.9 mN mm^{-2} to 6.2 ± 0.3 mN mm^{-2}) (Fig. 3F). Spontaneous recovery of net force was not seen for any hypercapnic cardiac strips. In dogfish (Fig. 3D), 50 mM HCO_3^- induced a partial but significant recovery of the hypercapnic cardiac net force to 9.2 ± 1.1 mN mm^{-2}, one that was mimicked NaOH additions. KH7 had no effect on the partial recovery, suggesting a pH-mediated, sAC-independent mechanism can partially rescue dogfish cardiac force during hypercapnia. HCO_3^- additions in the swamp eel did not change hypercapnic net force (Fig. 3E) and they significantly decreased hypercapnic net force

(6.2 ± 0.3 mN mm^{-2} to 4.2 ± 0.5 mN mm^{-2}) in the flounder (Fig. 3F). This decline was likely due to progressive deterioration of the preparation, as suggested for hypoxic flounder cardiac strips. Addition of NaOH or KH7 had no effect on hypercapnic net force in swamp eel and flounder.

Evidence for sAC-like immunostaining in lamprey heart

Since the effects of KH7 on lamprey heart suggested the involvement of sAC, we conducted immunostaining experiments using antibodies against sAC from the dogfish shark. sAC-like immunoreactivity was present throughout the heart of juvenile lampreys (Fig. 4A), which was absent when the primary antibodies were omitted (Fig. 4B) and in the peptide pre-absorption control (Supplementary Fig. 1).

Discussion

The regulation of fish cardiac function during hypoxia and hypercapnia by central and peripheral chemoreceptors acting through adrenergic and cholinergic pathways is well established (reviewed by Farrell and Smith 2017; Tresguerres et al. 2019). Here, we investigated whether HCO_3^- could directly modulate cardiac function in various fish species challenged with severe hypoxia or hypercapnic acidosis. Both challenges induced a significant decrease in heart rate in all tested species, which was expected based on the extensive literature described in the Introduction. The effect on heart rate of adding HCO_3^-, however, was both species- and challenge-specific.

Under our experimental conditions of severe hypercapnia and hypoxia, a HCO_3^--dependent, sAC-dependent rescue of heart rate was conclusively demonstrated only in lamprey hearts. When exposed to severe hypoxia, the ~30% drop in heart rate in isolated lamprey hearts

413 was completely recovered by addition of 10 mM HCO_3^- and heart rate significant overshot the
414 control level by ~15% with 30 mM HCO_3^-. This HCO_3^--dependent recovery was completely
415 abolished by the small molecule KH7, suggesting it was mediated by sAC. Similarly, Pacific
416 hagfish hearts exposed to severe hypoxia also demonstrated HCO_3^--dependent, sAC-dependent
417 recovery of heart rate (Wilson et al. 2016) and this transduction pathway may prove to be more
418 widespread among cyclostome fishes. A HCO_3^--dependent, sAC-dependent pathway was also
419 involved in increasing heart rate in isolated lamprey hearts exposed to severe hypercapnia. This
420 recovery, however, was partial (~50%) and required 40 mM HCO_3^-, which is much higher than
421 the 10 mM required to fully rescue heart rate during severe hypoxia. The reasons behind these
422 differences are not obvious, but could be related to a greater damaging effect of the experimental
423 hypercapnic acidosis, which reduced heart rate by almost 80% (while the hypoxia treatment
424 reduced heart rate by only ~30%). In addition, hypercapnia and hypoxia have different
425 mechanisms of impairment. For example, while intracellular acidification is common to both
426 treatments, it has different origins, namely an upregulation of anaerobic metabolism during
427 severe hypoxia and an inward CO_2 diffusion during severe hypercapnia. Consequently, future
428 research should investigate whether the HCO_3^--dependent, sAC-dependent transduction
429 mechanism can totally restore lamprey heart rate during a milder hypercapnic condition and
430 whether the lamprey heart can fully power routine cardiac performance with a glycolytic ATP
431 supply, as is the case for the hagfish heart (Farrell and Stecyk 2007; Cox et al. 2010; Cox et al.
432 2011). While we know little of the environmental levels of oxygen and carbon dioxide in the
433 sediments that some of these fish inhabit, hypoxia and hypercapnia often take place together in
434 natural environments (Jensen et al. 1993; Robinson 2019). Thus, future work should also
435 consider the potential interaction and effects of hypoxia and hypercapnia. Interestingly, the

436 [HCO_3^-] EC_{50} for recovery of the lamprey hypoxic heart rate (~5 mM) does match lamprey
437 plasma normal [HCO_3^-] values (Mattsoff and Nikinmaa 1988; Tufts and Boutilliet 1989), and the
438 [HCO_3^-] that sustained full recovery and overshoot (~10-30 mM) can be expected in plasma
439 during recovery from hypercapnia (Tresguerres et al. 2019; Wood 2019). While KH7 did not
440 have any cardiac effects in the other fish five species tested (which rules out non-specific KH7
441 toxicity due to off-target effects), we caution that investigation of a broader and less severe range
442 of hypoxia and hypercapnia is needed before we can eliminate a sAC-dependent regulation of the
443 heart in fish species other than cyclostomes.
444 The presence of sAC in the lamprey heart was further supported by an intense sAC-like
445 immunostaining using antibodies designed against an epitope in dogfish shark sAC (Tresguerres
446 et al. 2010). The combined pharmacological and immunohistochemical data provide solid
447 evidence about the presence and role of sAC in the lamprey heart. Definite demonstration of sAC
448 presence in cyclostomes, nonetheless, will require cloning of the sAC gene(s). Similarly, our
449 results do not rule out the presence of sAC in the heart of sturgeon, swamp eel, starry flounder,
450 or zebrafish. Unfortunately, obtaining definitive answers to these questions is not trivial because
451 the high complexity of sAC genes greatly confuses identification efforts using bioinformatic
452 approaches (Tresguerres and Salmerón 2018; Salmerón et al. in press).
453 Some of the fish hearts benefited from the stimulatory effects of HCO_3^- during severe
454 hypoxia and hypercapnic acidosis. These effects were insensitive to KH7 and therefore
455 independent from sAC activity. For example, addition of HCO_3^- during hypoxia enabled a total
456 recovery of heart rate in the dogfish and the swamp eel, but to do so required much higher
457 [HCO_3^-] compared to the lamprey. Importantly, addition of NaOH to mimic the increase in
458 external pH and Na^+ resulting from the addition of HCO_3^- had no effect on heart rate in any of

459 these species. Thus, HCO_3^- directly protected heart rate, possibly *via* transport into
460 cardiomyocytes by Na^+/HCO_3^- cotransporters or HCO_3^-/Cl^- exchangers for enhanced intracellular
461 pH regulatory capacity (Madshus 1988; Lagadic-Gossmann et al. 1992; Liu et al. 1990).
462 However, addressing this possibility question will require further experiments. On the other
463 hand, the hypoxia-induced reduction in heart rate for sturgeon, flounder and zebrafish was
464 unaffected by addition of HCO_3^-.

465 Addition of HCO_3^- also had a stimulatory effect during severe hypercapnic acidosis that
466 resulted in complete recovery of heart rate in dogfish, swamp eel, and sturgeon, and partial
467 recovery of heart rate in zebrafish. In these four species, the HCO_3^--mediated stimulation was
468 insensitive to KH7 and therefore sAC-independent. Intriguingly, the stimulatory effect of HCO_3^-
469 on isolated hearts exposed to hypercapnia was mimicked by addition of NaOH in dogfish and
470 sturgeon, but not in swamp eel and zebrafish. On the other hand, addition of HCO_3^- did not have
471 any effect on heart rate of flounder exposed to hypercapnic acidosis, as was the case with severe
472 hypoxia.

473 Experiments on isolated ventricular strips were possible with the three larger fish species,
474 the dogfish, the swamp eel, and the flounder. Unfortunately, hearts from juvenile lamprey,
475 sturgeon, and zebrafish proved too small for this technique. Our experiments confirmed a
476 debilitating effect of severe hypoxia and hypercapnia on ventricular net force. The only effect of
477 HCO_3^- addition was a partial recovery of net force in dogfish during hypercapnic acidosis. This
478 effect was mimicked by addition of NaOH, indicating a role for external pH or Na^+. The effect
479 was insensitive to KH7 and therefore was not mediated by sAC. Our results for ventricular strips
480 do differ from those reported in previous studies (Poupa and Johansen 1975; Gesser and Poupa
481 1979; Gesser and Jorgensen 1982; Gesser and Poupa 1983) in that we observed no spontaneous

recovery of cardiac net force during exposure to hypercapnic acidosis, or any protective effect of HCO_3^- in swamp eel or flounder. We do note that our study may not be directly comparable those earlier studies because their saline contained a high [HCO_3^-] (~30 mM) from the beginning of the exposure to 15% CO_2 (e.g. Gesser and Jorgensen 1982), while our exposure to hypercapnia started with a nominally zero HCO_3^- concentration.

The reasons behind the many species-specific differences observed in the present and previous studies are unclear. Some of the quantitative differences likely relate to the biological relevance of the levels of hypoxia, hypercapnic acidosis and [HCO_3^-] that were used, but it was important to standardize the experimental conditions as much as possible. Other differences may be explained by the absence of innervation or hormonal control in isolated preparations. For example, the hagfish heart receives no innervation, while the lamprey heart receives only vagal innervation (Nilsson 1983; Farrell and Smith 2017). Paracrine control of heart rate exists in both species, nevertheless, and the tonic release of catecholamines from intracardiac chromaffin tissue stores stimulate β-adrenergic receptors and increase cAMP levels in the heart (Ostlund et al. 1960; von Euler and Fange 1961; Axelsson et al. 1990; Wilson et al. 2016). We also performed preliminary work on β-adrenergic control of the normoxic lamprey heart, showing that isoproterenol nearly doubled heart rate (as did forskolin), a response blocked by propranolol (Supplementary Fig. 2 and 3). Future work should consider the possibility that sAC-mediated production of cAMP replaces a hypoxia-inactivated β-adrenergic one, as suggested by Wilson et al. (2016). In addition, the affinity of sAC for its substrate ATP is in the low millimolar range, and this affinity is much lower than that of tmAC. This opens the possibility that some of our results might be due not to the presence or absence of sAC, but to species-specific effects of our experimental conditions on intracellular ATP content. Indeed, sAC has been proposed to act as a

physiological ATP sensor in some mammalian cells (Zippin et al. 2013). This is another question that could be investigated through future research.

Immunofluorescence staining shows sAC presence in both the atrial and ventricular myocardium, and not just in the pacemaker region of lamprey and hagfish hearts (Wilson et al. 2016). Therefore, sAC could modulate multiple and diverse cardiac functions in these fishes. For example, mammalian sAC plays roles in the apoptosis of coronary endothelial cells and cardiomyocytes (Chen et al. 2011) and in modulating cardiac hypertrophic responses induced β-adrenergic and pressure overloads (Schirmer et al. 2018). sAC protein is also abundantly present in the hearts of other elasmobranch (Roa and Tresguerres 2017) and ray-finned fishes (Salmerón et al. in press). Very recently, HCO_3^-- mediated and sAC-mediated regulation of rat basal cardiac contractility was revealed based on the inhibitory effects of KH7 by analyzing sarcomere shortening in isolated cardiomyocytes combined with intracellular Ca^{2+} and pH measurements (Espejo et al. 2020). Similar sophisticated techniques may be required to further study HCO_3^--mediated and sAC-dependent mechanisms in fish.

In summary, the emerging picture about the effects of acid-base parameters on fish cardiac function is quite complex and species-specific. The current study supports the existence of a HCO_3^-- and sAC-dependent mechanism that rescues the heart rate of lamprey hearts exposed *in vitro* to hypoxia and hypercapnic acidosis. A similar mechanism was previously been described in isolated hagfish hearts during severe hypoxia (Wilson et al. 2016), and could apply to all cyclostomes. We found no evidence for a regulatory role of sAC on cardiac function of any of the other study species under our specific experimental conditions. However, HCO_3^- had species-, treatment-, and pH-independent protective effects that deserve further investigation. We hope the results presented here will serve as a baseline for future research, which could

investigate the effects of additional pharmacological agents and combinations of hypoxia, hypercapnia and [HCO_3^-], perhaps through more detailed studies in single species.

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Competing Interests

The authors declare no competing of financial interests

Author Contributions

M. L was involved in study conception and design, carried out all isolated heart experiments, data analysis, and produced the first draft. A. S. assisted with the lamprey isolated heart experiments. J. N. R. performed the microscopy work. M. T. designed and contributed to the microscopy work, data analysis, and manuscript editing. A. P. F. was involved in the study conception and design. All authors reviewed and revised the manuscript, and gave final approval for publication.

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744 **Table 1** Experimental conditions during severe hypoxia and hypercapnic acidosis. Values are mean ± s.e.m

	Dogfish	Flounder	Swamp Eel	Lamprey	Sturgeon	Zebrafish
Temperature (°C)	10	10	15	10	10	20
pH of control saline	7.8					
$[HCO_3^-]$ (mM) of control saline	0.9 ± 0.1	0.8 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.7 ± 0.1
Severe Hypoxia						
Duration of hypoxia (h)	1	1	1	2	1	0.5
pH of saline with N_2 + 50 mM $NaHCO_3$	8.7 ± 0.1	8.7 ± 0.1	8.7 ± 0.1	8.7 ± 0.1	8.7 ± 0.1	8.7 ± 0.1
$[HCO_3^-]$ (mM) of saline with N_2 + 50 mM $NaHCO_3$	49 ± 2	47 ± 1	43± 1	43 ± 1	43 ± 1	43 ± 1
Hypercapnic Acidosis						
% CO_2	15%	15%	15%	7.5%	7.5%	7.5%
pH of saline with CO_2	6.0 ± 0.1	5.9 ± 0.1	5.9 ± 0.1	6.1 ± 0.1	6.1 ± 0.1	6.1 ± 0.1
$[HCO_3^-]$ (mM) of saline with CO_2	2.6 ± 0.3	2.1 ± 0.3	2.3 ± 0.3	1.9 ± 0.2	1.9 ± 0.2	2.2 ± 0.2
pH of saline with CO_2 + 50 mM $NaHCO_3$	7.1 ± 0.1	7.2 ± 0.1	7.2 ± 0.1	7.5 ± 0.1	7.5 ± 0.1	7.5 ± 0.1
$[HCO_3^-]$ (mM) of saline with CO_2 + 50 mM $NaHCO_3$	49 ± 2	48 ± 2	49 ± 2	47 ± 2	47 ± 2	50 ± 2

745

746

747 **Fig. 1** Effects of cumulative $NaHCO_3$ addition on the beat rate of isolated hearts during severe
748 hypoxia. (A) Lamprey. (B) Dogfish. (C) Swamp Eel. (D) Sturgeon. (E) Flounder. (F) Zebrafish.
749 Red square: KH7 + $NaHCO_3^-$ (40 mM for lamprey, 50 mM for the other species). Green triangle:
750 NaOH + no $NaHCO_3$. Inverted blue triangle: DMSO + 40 mM $NaHCO_3^-$ (only in lamprey).
751 Different letters indicate statistically significant differences within the $NaHCO_3$ and $NaHCO_3$ +
752 KH7 values (one-way repeated measures ANOVA). Asterisks indicate statistically significant
753 differences between the NaOH or DMSO treatment with the highest [$NaHCO_3$] (one-way
754 ANOVA). Data is shown as normalized values relative to the normoxic heart rate (mean ±
755 s.e.m.; where not visible they fall within the symbol of the data point); statistical analyses were
756 done on the raw data. $P<0.05$ (n=6)

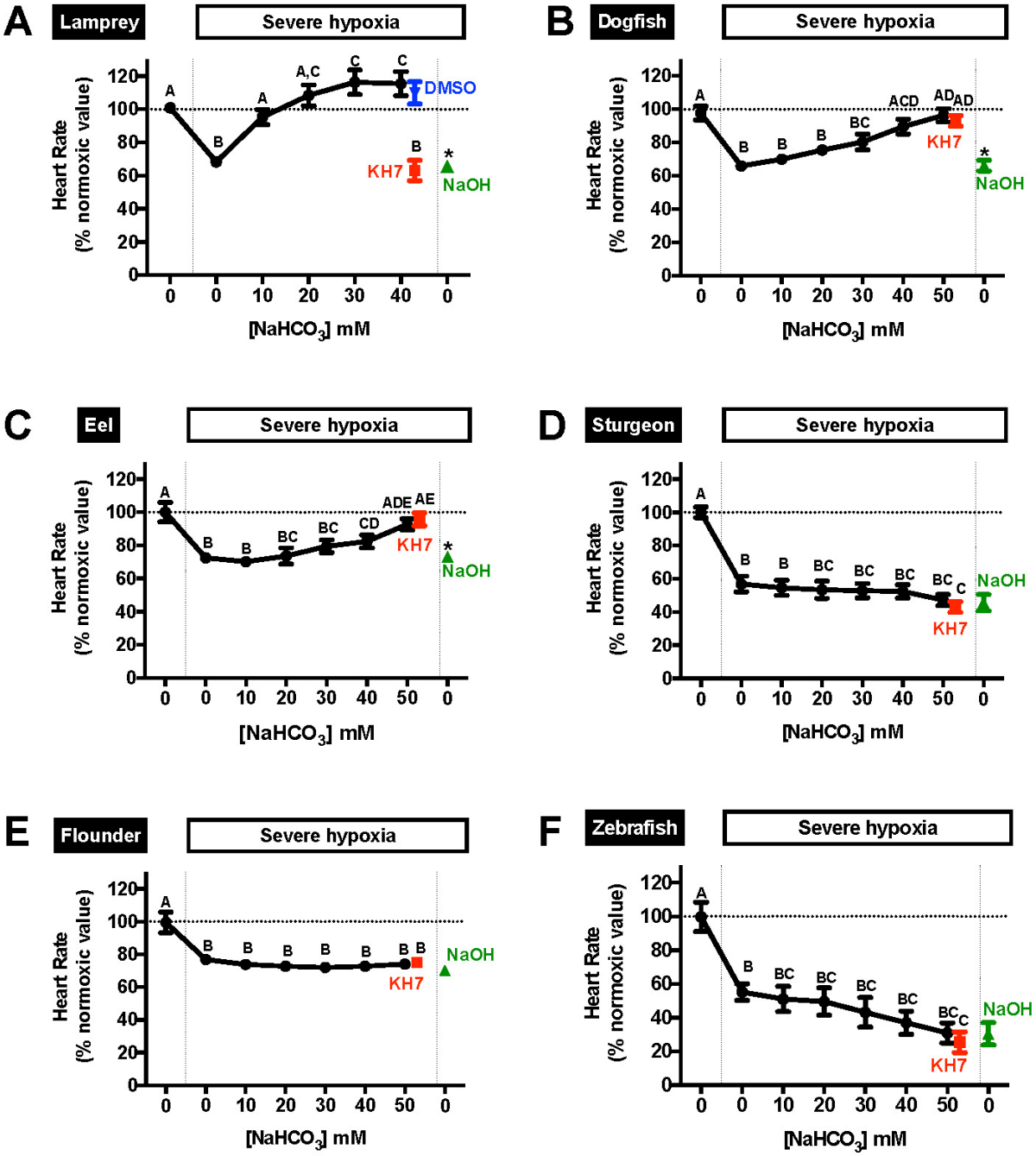
757

758 **Fig. 2** Effects of cumulative $NaHCO_3$ addition on the beat rate of isolated hearts during
759 hypercapnic acidosis. (A) Lamprey. (B) Dogfish. (C) Swamp Eel. (D) Sturgeon. (E) Flounder.
760 (F) Zebrafish. Red square: KH7 + $NaHCO_3^-$ (40 mM for lamprey, 50 mM for the other species).
761 Green triangle: NaOH + no $NaHCO_3$. White circle: hearts at the end of the hypercapnic exposure,
762 without $NaHCO_3$ addition. Inverted blue triangle: DMSO + 40 mM $NaHCO_3^-$ (only in lamprey).
763 Different letters indicate statistically significant differences within the $NaHCO_3$ and $NaHCO_3$ +
764 KH7 values (one-way repeated measures ANOVA). Asterisks indicate statistically significant
765 differences between the NaOH, DMSO, or control treatment with the highest [$NaHCO_3$] (one-
766 way ANOVA). Data is shown as normalized values relative to the normoxic heart rate (mean ±
767 s.e.m., where not visible they fall within the symbol of the data point); statistical analyses were
768 done on the raw data. $P<0.05$ (n=6)

Fig. 3 Effects of cumulative $NaHCO_3$ additions on the contractility of cardiac strips during severe hypoxia and hypercapnic acidosis. (A, D) Dogfish. (B, E) Swamp Eel. (C, F) Flounder. Red square: KH7 + $NaHCO_3^-$. Green triangle: NaOH + no $NaHCO_3$. White circle: hearts at the end of the hypercapnic exposure, without $NaHCO_3$ addition. Different letters indicate statistically significant differences within the $NaHCO_3$ and $NaHCO_3$+ KH7 values (one-way repeated measures ANOVA). Asterisks indicate statistically significant differences between the NaOH or control treatment with the highest [$NaHCO_3$] (one-way ANOVA). Data is shown as normalized values relative to the normoxic or initial contractility (mean ± s.e.m., where not visible they fall within the symbol of the data point); statistical analyses were done on the raw data. $P<0.05$ (n=6)

Fig. 4 sAC-like immunoreactivity in lamprey heart. (A) sAC-like immunostaining (green) is evident throughout the heart. (B) Omission of primary antibody control, where only secondary antibodies were applied. Nuclei stained with Hoechst 33342 appear in blue

792 Figure 1



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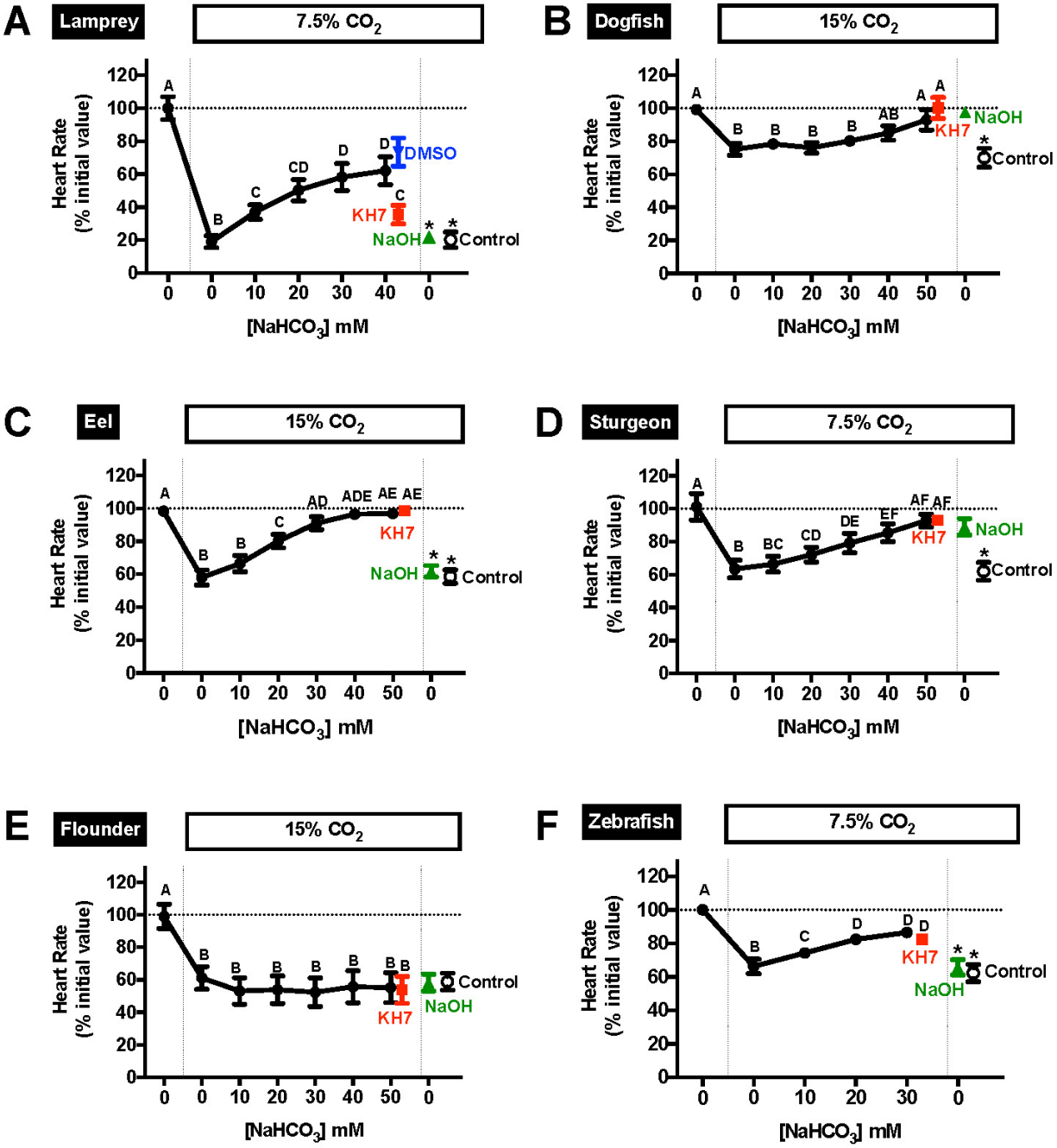
797 Figure 2



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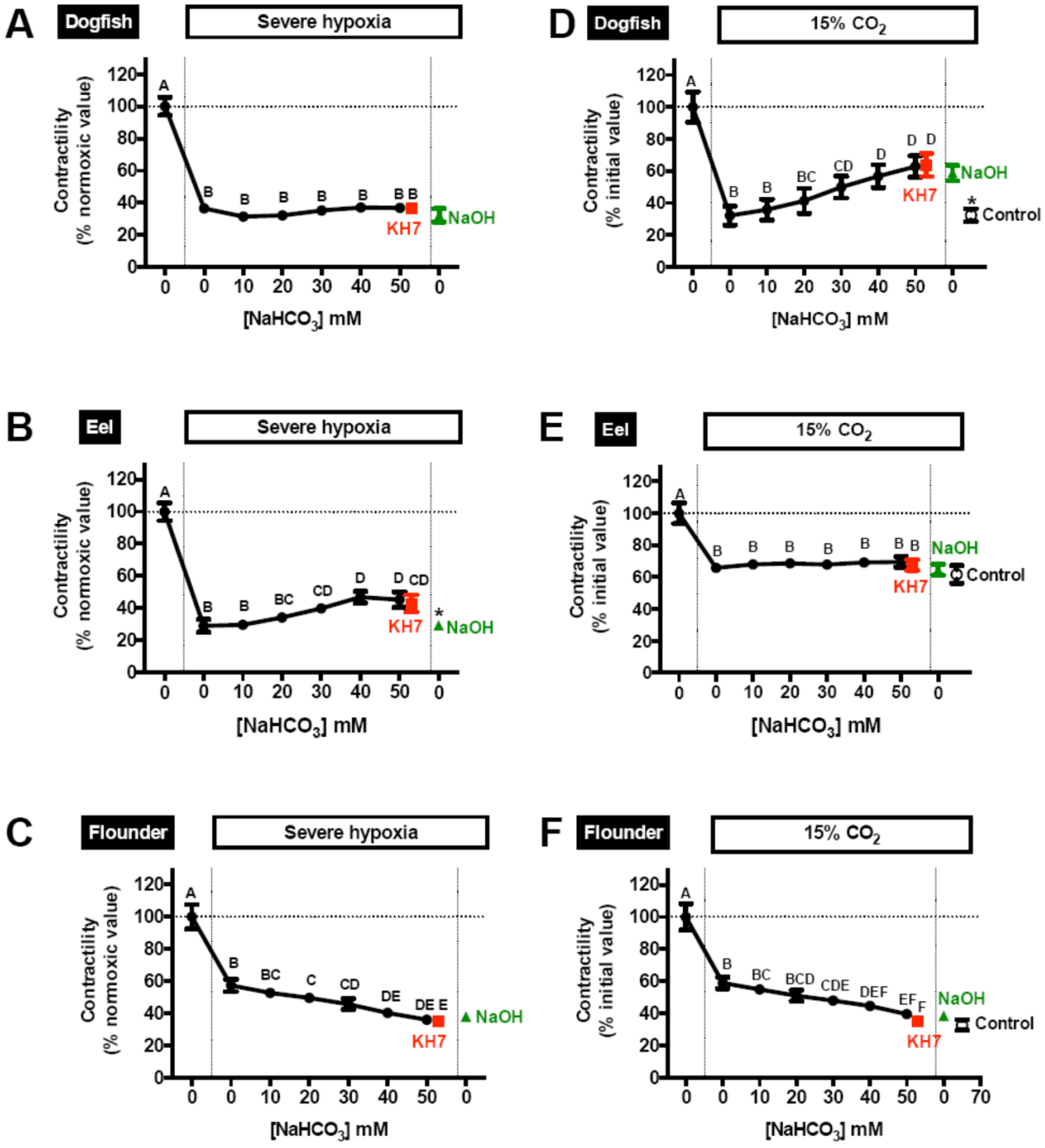
802 Figure 3



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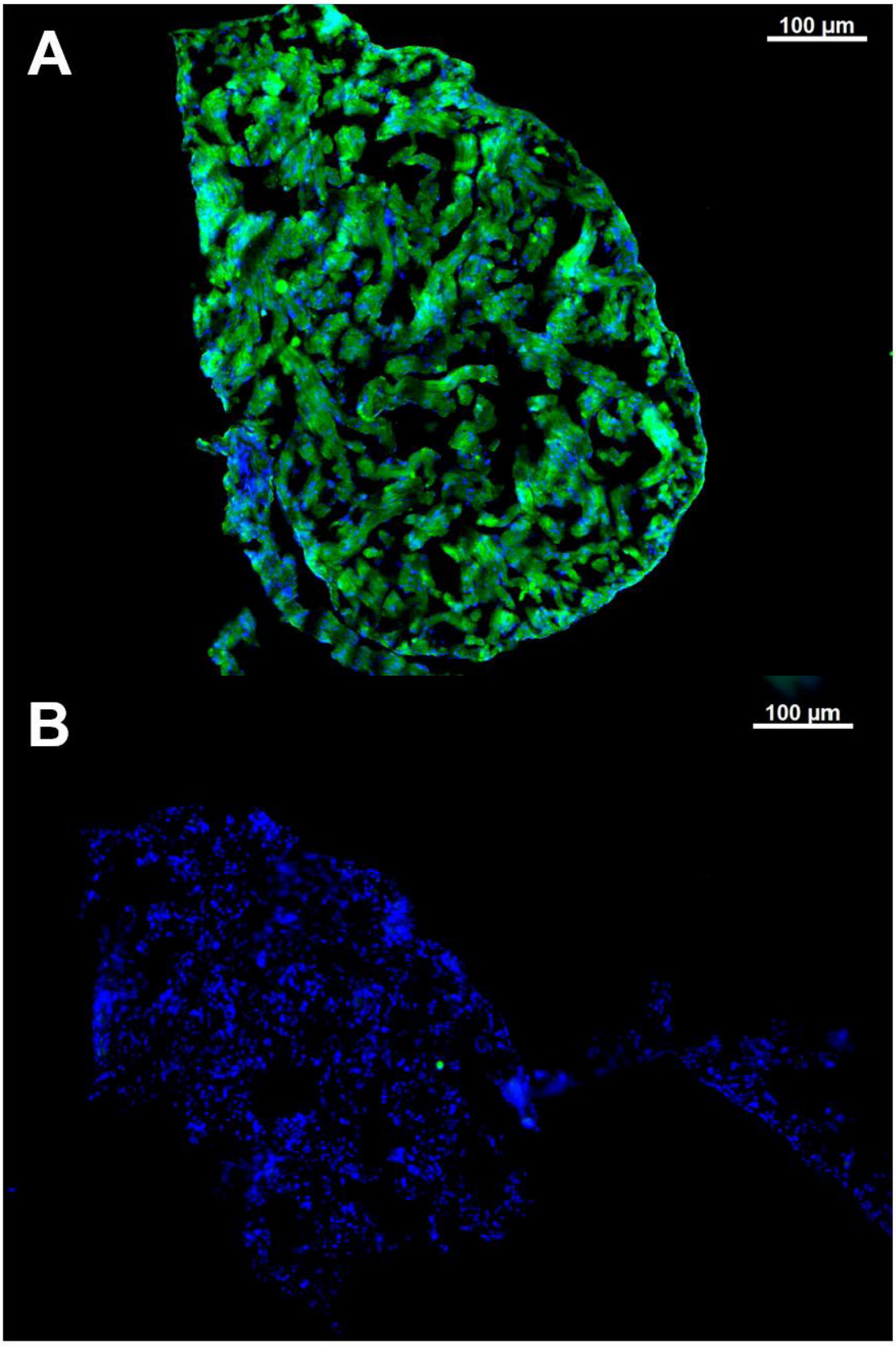
808 Figure 4



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