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RESEARCH ARTICLE

Evidence for a role of heme oxygenase-1 in the control of cardiac function in zebrafish (*Danio rerio*) larvae exposed to hypoxia

Velislava Tzaneva* and Steve F. Perry

ABSTRACT

Carbon monoxide (CO) is a gaseous neurotransmitter produced from the breakdown of heme via heme oxygenase-1 (HO-1; hypoxia-inducible isoform) and heme oxygenase-2 (HO-2; constitutively expressed isoform). In mammals, CO is involved in modulating cardiac function. The role of the HO-1/CO system in the control of heart function in fish, however, is unknown and investigating its physiological function in lower vertebrates will provide a better understanding of the evolution of this regulatory mechanism. We explored the role of the HO-1/CO system in larval zebrafish (*Danio rerio*) *in vivo* by investigating the impact of translational gene knockdown of HO-1 on cardiac function. Immunohistochemistry revealed the presence of HO-1 in the pacemaker cells of the heart at 4 days post-fertilization and thus the potential for CO production at these sites. Sham-treated zebrafish larvae (experiencing normal levels of HO-1) significantly increased heart rate (f_H) when exposed to hypoxia (Pw_{O_2}=30 mmHg). Zebrafish larvae lacking HO-1 expression after morpholino knockdown (morphants) exhibited significantly higher f_H under normoxic (but not hypoxic) conditions when compared with sham-treaded fish. The increased f_H in HO-1 morphants was rescued (f_H was restored to control levels) after treatment of larvae with a CO-releasing molecule (40 µmol l^{-1} CORM). The HO-1-deficient larvae developed significantly larger ventricles and when exposed to hypoxia they displayed higher cardiac output ($\dot{Q}$) and stroke volume (SV). These results suggest that under hypoxic conditions, HO-1 regulates $\dot{Q}$ and SV presumably via the production of CO. Overall, this study provides a better understanding of the role of the HO-1/CO system in controlling heart function in lower vertebrates. We demonstrate for the first time the ability for CO to be produced in presumptive pacemaker cells of the heart where it plays an inhibitory role in setting the resting cardiac frequency.

KEY WORDS: Heart size, Carbon monoxide, Heart rate, Pacemaker cells

INTRODUCTION

Low environmental oxygen (O_2) levels, also known as hypoxia, are a common occurrence in a variety of aquatic environments (Diaz and Breitburg, 2009). Adult water-breathing fish exposed to hypoxia exhibit well-defined cardiorespiratory responses consisting of reflex hyperventilation (Dejours, 1973; Randall et al., 1976; Perry and Gilmour, 1996; Vulesevic et al., 2006; Tzaneva et al., 2011) and bradycardia (Randall and Shelton, 1963; Randall and Smith, 1967; reviewed in Farrell, 2007 and Gamperl and Shiels, 2014). Hypoxic bradycardia is usually accompanied by an increase in stroke volume (SV) such that cardiac output ($\dot{Q}$) is maintained (Holeton and Randall, 1967; Short et al., 1979; Wood and Shelton, 1980; Fritsche and Nilsson, 1990). Although bradycardia is the typical response to hypoxia in adult water-breathing fish, the situation is much less clear in developing larvae. Zebrafish (*Danio rerio*) larvae, in particular, exhibit various cardiac responses depending on the severity of hypoxia and/or the duration of exposure (acute versus chronic) (Barrionuevo and Burggren, 1999; Jacob et al., 2002; Yaqoob and Schwerte, 2010; Bagatto, 2005; Steele et al., 2011). Tachycardia may be the initial response to progressive hypoxia but can develop into bradycardia as the severity or duration of the hypoxia is increased. For example, Jacob et al. (2002) reported significant tachycardia in 5 day old zebrafish larvae exposed to moderate levels of hypoxia (~75 mmHg or roughly 4 mg l^{-1} dissolved O_2). In a similar experiment, however, Bagatto (2005) demonstrated that zebrafish larvae developed a pronounced bradycardia in response to severe hypoxia (the reported level of dissolved O_2 was 0.8 mg l^{-1}). Steele and colleagues (2011) also observed a decrease in cardiac frequency (f_H) of zebrafish larvae exposed to severe hypoxia (~20 mmHg or roughly 1.0 mg l^{-1} dissolved O_2) at 5–7 days post-fertilization (dpf). In one specific instance, hypoxic bradycardia in larval zebrafish (6 dpf) was accompanied by an elevation of $\dot{Q}$ owing to disproportionally increased SV (Yaqoob and Schwerte, 2010). Hypoxic tachycardia is also a typical response of larval Arctic char (*Salvelinus alpinus*) (McDonald and McMahon, 1977) and trout (*Oncorhynchus mykiss*) (Holeton, 1971; McDonald and McMahon, 1977).

Previous studies on hypoxic modulation of cardiac function in adult teleost fish have focused on the involvement of the autonomic nervous system (Wood and Shelton, 1980; Burleson and Milsom, 1993; Stecyk and Farrell, 2006). These studies have demonstrated that low O_2 levels result in cholinergically mediated bradycardia (Wood and Shelton, 1980; Burleson and Milsom, 1993; Stecyk and Farrell, 2006). In larval zebrafish, the onset of cardiac control via the parasympathetic and sympathetic nervous systems (or circulating catecholamines) is evident as early as 4 dpf (Steele et al., 2009, 2011). The development of bradycardia in the face of chronic hypoxia in zebrafish larvae is mediated by cardiac muscarinic receptors (Bagatto, 2005) of the M_2 sub-type (Steele et al., 2009), while the tachycardia response to acute hypoxia is probably controlled by a release of inhibitory vagal tone or stimulation of adrenergic receptors by elevated circulating catecholamine levels or catecholamines released from sympathetic neurons (Steele et al., 2009, 2011).

Although there is evidence of autonomic nervous system control of heart function in fish, more recent models of cardiac modulation during acute hypoxia incorporate the involvement of gasotransmitters such as carbon monoxide (CO) (Durante et al., 2006). In fish, endogenous CO is involved in controlling several physiological functions including ventilation (Tzaneva and Perry,

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2014) and f_H (reviewed in Olson and Donald, 2009; Perry et al., 2016). Endogenous CO is produced from the breakdown of heme into biliverdin, Fe^{2+} and CO by the heme oxygenase (HO) family of enzymes. Biliverdin is further reduced to bilirubin by biliverdin reductase (reviewed in Abraham and Kappas, 2008). Both biliverdin and bilirubin are reducing molecules and act as scavengers for reactive oxygen species (ROS), thereby reducing oxidant-mediated cell death (Kushida et al., 2002; reviewed in Abraham and Kappas, 2008). Fe^{2+} generated from the HO-catalysed degradation of heme can result in the formation of ROS, which can damage various cellular components. Thus, it is sequestered by ferritin induced by Fe^{2+} release and is eventually recycled as a substrate for hemoglobin synthesis (Sassa, 2006). Two isoforms of the HO enzyme have been identified in zebrafish – the hypoxia-inducible heme oxygenase-1 (HO-1) and the constitutively expressed heme oxygenase-2 (HO-2) (Thisse and Thisse, 2004; Voelker et al., 2008). CO cannot be stored in vesicles and thus upon synthesis, it diffuses readily across membranes to evoke appropriate physiological responses in an autocrine/paracrine manner (Levitt and Levitt, 2015).

Relatively few studies have investigated the expression of HO-1 and its activity in teleost species although zebrafish have received the most attention. HO-1 mRNA was detected in the red blood cells of wild-type zebrafish larvae as early as 34 h post-fertilization (Craven et al., 2005) and expression in whole larvae was shown to increase with exposure to the toxicant 3,4-dichloroaniline (Voelker et al., 2008). More recently, a significant increase in HO-1 mRNA was reported in the hearts of adult zebrafish exposed to 9 h of hypoxia (Parente et al., 2013). Although HO-1 is known to increase in zebrafish under toxic (e.g. exposure to 3,4-dichloroaniline; Voelker et al., 2008) or hypoxic conditions, little is known about its role in regulating cardiac function at such times. In the mammalian heart, the HO-1/CO system maintains ventricular size (volume and muscle mass) and blood pressure during acute hypoxia (Chen et al., 2003; Hartsfield et al., 2004). Furthermore, several lines of evidence suggest that HO-1 exerts cytoprotective and anti-apoptotic effects on mammalian cardiomyocytes via CO (Abraham and Kappas, 2008). In contrast to acute hypoxia, where increases in HO-1 activity but not protein levels are seen, chronic hypoxia causes a progressive increase in both activity and protein levels of HO-1 within the first 14 days of exposure in the hearts of rats (Grilli et al., 2003). Furthermore, the highest activity of the non-inducible HO-2 was detected in the brain. CO produced via HO-2 functions as a signalling molecule in the central nervous system and in the circulatory system (reviewed in Olas, 2014).

The present work focuses on zebrafish, a cyprinid species that has been used widely as a model organism to study cardiac function (reviewed in Bakkers, 2011). We specifically aimed to link endogenously produced CO via HO-1 to control of heart function in developing larvae. We hypothesized that the hypoxia-inducible HO-1/CO system plays an integral role in controlling heart function in zebrafish larvae during acute hypoxia. Measurements of f_H, SV and $\dot{Q}$ were complemented with immunohistochemistry and HO-1 enzyme activity measurements to determine the location of HO-1 and the potential for endogenous CO production in the heart of zebrafish larvae.

MATERIALS AND METHODS

Zebrafish husbandry

Adult zebrafish, *Danio rerio* (Hamilton 1822) were bred and raised at the University of Ottawa aquatic care facility, where they were kept in 10 l acrylic tanks in multi-rack flow-through aquatic housing systems (Aquatic Habitats, Apopka, FL, USA). The tanks were supplied with aerated dechloraminated City of Ottawa tap water maintained at 28°C under a 12:12 h light:dark cycle. To obtain embryos, one male was paired with two females in a 2 l tank with a separator dividing males and females into separate compartments. After an overnight acclimation period, the water was changed and the separator was lifted, allowing spawning to proceed for at least 15 min.

Injection of HO-1 morpholino

The embryos were collected and injected with an antisense morpholino oligonucleotide (5′-AGTCCATCTTTGTGCTGTAG-ATGTC-3′) designed to bind to the translation start site of zebrafish HO-1 (UniProt accession number B0UXS0-1) and prevent translation of the HO-1 protein. Embryos were injected at the one-cell developmental stage, which lasts 15–30 min post-fertilization (Kimmel et al., 1995). The working stock morpholino was diluted prior to injection in 1× Danieu buffer [58 mmol l^{-1} NaCl, 0.7 mmol l^{-1} KCl, 0.4 mmol l^{-1} $Ca(NO_3)_2$, 5.0 mmol l^{-1} Hepes (pH 7.6)] and 0.05% Phenol Red. The working concentration of the morpholino was 4 ng nl^{-1}. Approximately 1 nl was injected at the border between the yolk sack and the cell of each embryo to facilitate entry of the morpholino into the cell. The morpholino dose was validated by calibrating the microinjection needles used to disperse a constant droplet volume. The volume of the droplet was set to 1 nl by adjusting the microinjector pressure and time settings for each microinjection needle. To control for any effects of injecting nucleotide, matching concentrations of a control (sham) morpholino (5′-CCTCTTACCTCAGTTACAATTTATA-3′; Gene Tools, LLC, Philomath, OR, USA) were also administered to control (sham-treated) embryos; there are no known targets for this morpholino in zebrafish. Injected embryos were transferred to 300 ml Petri dishes containing system water with 0.03% Methylene Blue and incubated at 28°C for 4 days, at which time experiments were performed.

f_H and size measurements

Morphant and sham-treated larvae were placed individually for the duration of the experiments in temperature-controlled perfusion chambers with a maximum volume of 200 µl. All treatments contained 0.05 mg ml^{-1} Tris-buffered MS-222 (ethyl-3-aminobenzoate methanesulphonate salt, Sigma-Aldrich, Oakville ON, Canada) and all experiments were performed at 28°C. The dose of 0.05 mg ml^{-1} of MS-222 was chosen because it was the lowest dose able to keep the larvae immobilized. Other studies have shown that high doses of MS-222 decrease f_H in zebrafish larvae; however, the concentration used in this study is lower than that used in those studies (Steele et al., 2009, 2011). Furthermore, in our own experiments only a dose of MS-222 above 0.12 mg ml^{-1} caused a decrease in f_H after 10–15 min exposure of 4 dpf larvae. Therefore, we are confident that the concentration of MS-222 used in the current study did not cause any non-specific effects on f_H during the experiments. Each larva was exposed to normoxia for 3 min to obtain a baseline f_H, after which the water flow was switched for 15 min to normoxic water containing either 40 µmol l^{-1} carbon monoxide-releasing molecule (CORM; SML0496, Sigma-Aldrich) or 10^{-4} mol l^{-1} adrenaline [general adrenergic receptor agonist; (±)-epinephrine hydrochloride, E4250, Sigma-Aldrich]. In a separate group of fish, larvae were exposed to normoxia for 3 min to obtain a baseline f_H, after which the water flow was switched to hypoxic water (partial pressure of O_2 in the water, Pw_{O_2}=30 mmHg) for 15 min. Hypoxia was achieved by bubbling a gas mixture of 85% N_2 and 15% air continuously into a water reservoir maintained at 28°C to maintain a stable Pw_{O_2} of approximately 30 mmHg. Following the hypoxic treatment, larvae were exposed to hypoxic water

containing either 40 µmol l^{-1} CORM or 10^{-4} mol l^{-1} adrenaline for an additional 15 min. The adrenaline concentration used was based on a previous study (Steele et al., 2011) and preliminary trials which demonstrated that it was the lowest concentration to reliably produce a positive cardiac chronotropic effect. The CORM concentration was chosen based on dose–response curves performed with concentrations used previously in mammals (Clark et al., 2003). One group of control (non-injected) larvae was exposed to the same hypoxia treatment to ensure that there was no effect of injection on f_H in sham-treated larvae under normoxic conditions and that they did not exhibit an altered response to hypoxia (Fig. S1).

To measure f_H, SV and $\dot{Q}$, each trial (one larva per trial) was recorded continuously using a high-speed video camera (3CCD camera, Dage-MTI, USA) mounted on a dissecting microscope (CMZ1500, Nikon, USA) and connected to a personal computer. Videos were captured using CyberLink Power Director Software (Santa Clara, CA, USA) at 720×480 pixels and 30 frames s^{-1}. Heart beats were counted for 30 s and heart size was measured by taking a single photo of the heart and using the formula for an ellipsoid to determine heart volume. SV and $\dot{Q}$ were measured/calculated from the captured images as per Kopp et al. (2007).

HO-1 activity assay

The HO-1 activity assay was performed as described in McCoubrey (2001). Briefly, tissue samples (~30 larvae per sample) underwent two freeze–thaw cycles before homogenization in ice-cold homogenization buffer (1 mol l^{-1} Tris HCl, 250 mmol l^{-1} sucrose). The homogenized tissue was centrifuged at 12,000 ***g*** for 30 min at 4°C and the crude supernatant was collected from the lysates. The supernatant was incubated in the dark in a solution containing 18 µmol l^{-1} heme, 30 µl of 30 U ml^{-1} biliverdin reductase (BVR; Abcam Inc., Toronto, ON, Canada) and 2.75 mmol l^{-1} NADPH for 30 min at 37°C on a shaker. The reaction was stopped on ice and the concentration of bilirubin was calculated using the difference of absorbance between 470 and 530 nm using an extinction coefficient of 40 l^{-1} mol^{-1} cm^{-1}. Heme oxygenase activity was calculated as pmol bilirubin min^{-1} mg^{-1} protein.

Western blots and protein quantification

Frozen tissue samples (~30 larvae per sample) were thawed on ice and homogenized in 200 µl RIPA buffer (150 mmol l^{-1} NaCl, 1% Triton X-100, 0.5 sodium deoxycholate, 0.1% SDS, 50 mmol l^{-1} Tris HCl, 1 mmol l^{-1} EDTA, 1 mmol l^{-1} phenylmethanesulphonyl fluoride) with a protease inhibitor cocktail tablet (1 tablet per 10 ml RIPA buffer; Roche, USA). A bicinchoninic acid (BCA) assay was used to determine protein content and approximately 50 µg of total protein was used for western blotting. Protein samples were separated using a 12% TGX Stain-Free FastCast acrylamide gel (Bio-Rad, USA). After protein separation, the gel was activated for 1 min under UV light to visualize total protein before transfer. The separated samples were then transferred to a PVDF membrane (Bio-Rad, USA) and total protein on the membrane was visualized. The membrane was then blocked in 5% milk for 2 h at room temperature after which it was incubated overnight at 4°C with a primary zebrafish anti-HO-1 antibody (immunogen peptide MDSTKSKAAENTGSC) raised in rabbit (GenScript, USA) targeting the N-terminus of the HO-1 protein at 1:4000 dilution. The membrane was washed then probed with goat anti-rabbit secondary antibody (1:5000; Pierce, USA) for 2 h at room temperature. The amount of protein was represented as the fold change by measuring the intensity of the detected HO-1 band and total protein for each sample using ImageLab software (Bio-Rad, USA) and normalizing it against a pooled protein sample.

Immunohistochemistry

Morphant and sham larvae were killed using MS-222 and fixed in 4% paraformaldehyde for 2 h at room temperature. Larvae were immersed for 24 h in 30% sucrose then embedded in OCT Cryomatrix (Thermo Scientific, USA) after which they were sectioned longitudinally into 50 µm slices using a Leica CM3050 cryostat at −25°C (Leica Microsystems Inc.). The sections were incubated with 0.8% Triton X-100 in phosphate-buffered saline (PBS; pH 7.4) for 2 h. HO-1 immunoreactive cells were identified with an antibody against HO-1 (described above) and pacemaker cells of the heart were identified using an anti-mouse islet 1 (Isl1) antibody (Developmental Studies Hybridoma Bank, University of Iowa, Iowa; Tessadori et al., 2012). The primary antibodies were added to 1× PBS to yield final dilutions of 1:200 each. The sections were incubated with the primary antibodies overnight at room temperature. Secondary antibodies [Alexa 488 (green) and Alexa 594 (red)] were applied and sections were incubated in the dark for 2 h at room temperature. Sections were imaged using an upright confocal microscope (A1R MP+ Multiphoton Nikon Instruments Inc., USA) equipped with argon (peak output, 488 nm) and helium–neon (peak output, 543 nm) lasers. Optical slices of 1 µm were taken to produce an image using NIS Elements AR Viewer software (Nikon Instruments Inc., USA).

Statistical analyses

Data are represented as means±s.e.m. One-way and two-way ANOVA and two-way repeated-measures ANOVA were used to reveal significant differences between means with a Tukey *post hoc* test when significant differences were detected. The limit of significance was 0.05 for all analyses. Sigma Plot v12.0 (SPSS Inc.) was used to perform all statistical analyses.

RESULTS

HO-1 in the heart and pacemaker cells of zebrafish larvae

Three or four sections from each of 5 larvae at 4 dpf were examined to determine the presence of HO-1 in the heart of sham-treated and morphant fish. HO-1 immunoreactive cells were found in the atrium and ventricle of sham-treated fish (Fig. 1B) but were apparently absent (not observed) in HO-1 morphant fish (Fig. 2B). Furthermore, HO-1 appeared to be present within pacemaker (Isl-positive) cells of the ventricle and atrium of sham-treated, but not morphant fish (Figs 1C and 2C). Two sections of 4–5 sham-treated larvae at 4 dpf were used as negative controls on which the primary HO-1 antibody was omitted; no HO-1 immunoreactivity was detected (data not presented).

HO-1 activity assay

In sham-treated 4 dpf larvae exposed to acute hypoxia (15 min of Pw_{O_2}=30 mmHg), HO activity increased significantly from 27.3±1.8 to 41.9±5.3 pmol bilirubin min^{-1} mg^{-1} protein (two-way ANOVA, P=0.006; Fig. 3A). There was no significant difference detected in HO activity between normoxic morphant and sham-treated larvae (two-way ANOVA, P=0.433; Fig. 3A). Zebrafish morphant larvae failed to exhibit a significant increase in HO activity when exposed to acute hypoxia (two-way ANOVA, P=0.93; Fig. 3A).

HO-1 knockdown confirmation: western blots and protein quantification

Western blots using protein extracted from 4 dpf normoxic sham-treated larvae showed a clear band at ~32 kDa which was absent in normoxic HO-1 morphant larvae (insert in Fig. 3B). Hypoxia exposure did not cause a significant increase in HO-1 protein

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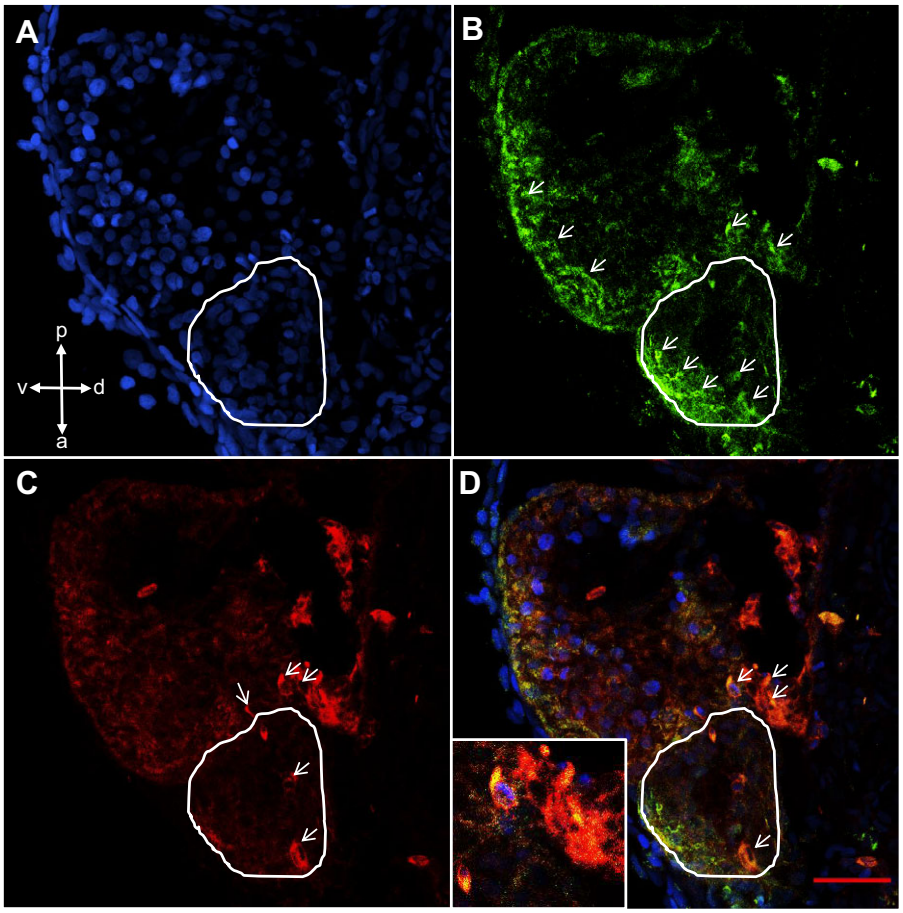

Fig. 1. Micrographs depicting a 50 µm cross-section of the heart of a 4 dpf sham-treated zebrafish larva. (A) The ventricle is indicated with a white outline and cells have been treated with DAPI to stain nuclei. a, anterior; d, dorsal; p, posterior; v, ventral. (B,C) Cells positive for heme oxygenase-1 (HO-1; B) and islet 1 (Isl1; C) are indicated with arrows. (D) Co-localization of HO-1 and Isl1, indicated with arrows. Inset in D represents a magnified view of HO-1- and Isl1-positive cells.

expression in either sham-treated or HO-1 morphant larvae although there was a trend for an increase in HO-1 protein content during hypoxia in sham-treated larvae (two-way ANOVA, O_2 treatment, P=0.07; Fig. 3B). HO-1 morpholino injections significantly diminished HO-1 protein content by ∼22-fold in normoxic morphant larvae when compared with sham-treated larvae, and in hypoxic morphant larvae by ∼19-fold, thus confirming knockdown of the HO-1 protein by the morpholino injections (Fig. 3B).

The effect of HO-1 on f_H, heart size, SV and $\dot{Q}$ in zebrafish larvae

The f_H of sham-treated fish did not differ significantly from that of non-injected control fish under normoxic conditions and in their response to acute hypoxia (Fig. S1). Based on this evidence, we are confident that the sham procedure did not affect baseline f_H and the ability of sham-treated fish to respond to hypoxia. Therefore, all subsequent experiments compared results from sham-treated and morpholino-injected fish.

Baseline f_H of HO-1 morphant fish was significantly higher than that in sham-treated fish (Fig. 4A). Hypoxia significantly increased the f_H of sham-treated fish from 144.1±3.9 to 155.0±6.0 beats min^{-1} but was without effect in morphant fish (Fig. 4A). Because f_H was already elevated in the normoxic HO-1 morphant fish, it raised the question as to whether HO-1 morphant fish have the capacity to increase their f_H further during hypoxia (i.e. f_H may have reached its maximal level). To test this idea, fish were exposed to the cardiac stimulant adrenaline. Adrenaline exposure caused an increase in f_H in sham-treated fish (158.7±3.4 to 197.0±4.0 beats min^{-1}) and morphant fish (180.0±4.1 to 196.0±6.7 beats min^{-1}) exposed to normoxia (Fig. 4B). Exogenous CO significantly decreased the f_H in both groups of fish under normoxic or hypoxic conditions (Fig. 4C,D).

Ventricular size is another indicator of heart function because it determines, at least in part, the amount of blood that is pumped from the heart during systole. A double-blind design was used to measure the ventricular volume of larvae. The measurements of ventricular volume (Fig. 5A) were performed on terminally anesthetized (MS-222), fixed 4 dpf zebrafish larvae. MS-222 causes depression of cardiac contractility at higher doses in larval fish and thus the ventricles of these fish were probably arrested and measured in a diastolic state (Denvir et al., 2008). HO-1 morphant fish exhibited a significantly larger diastolic ventricular volume (1.1±0.23 nl) compared with the sham-treated fish (0.5±0.06 nl) (Fig. 5A). Hypoxia caused a significant decrease in diastolic volume but not systolic volume in sham-treated fish only (Table 1). Systolic volume did not change significantly in either sham-treated or morphant fish when exposed to hypoxia (Table 1). There was no significant difference in the SV or $\dot{Q}$ between sham-treated and HO-1 morphant fish under normoxic conditions, but when exposed to hypoxia, morphant fish exhibited a significantly higher SV and $\dot{Q}$ (Fig. 5B,C).

DISCUSSION

The overall aim of this study was to investigate the role of the HO-1/CO system in the control of heart function in the developing zebrafish larva. Three major findings emerged: (i) HO-1 was found to be present in the pacemaker cells of the heart; (ii) lack of HO-1 resulted in significantly higher f_H under normoxic but not hypoxic conditions; and (iii) HO-1-deficient fish exhibited significantly larger ventricles

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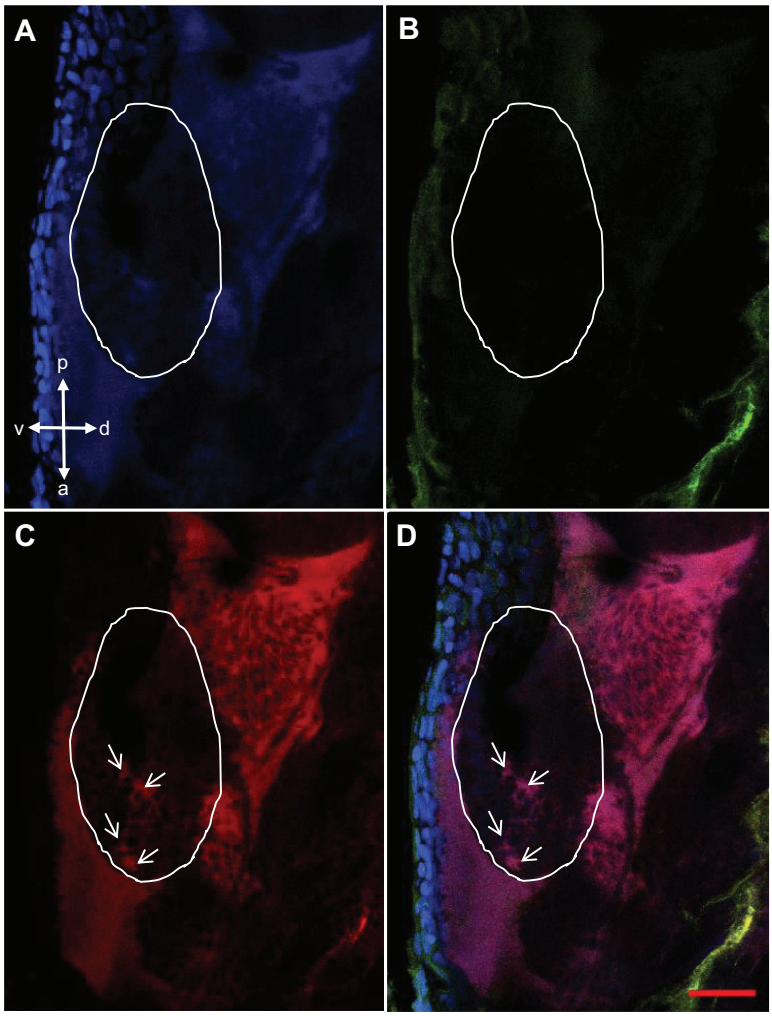

Fig. 2. Micrographs depicting a 50 µm cross-section of the heart of a 4 dpf HO-1 morphant zebrafish. (A) The ventricle is indicated with a white outline and cells have been treated with DAPI to stain nuclei. (B) HO-1 immunoreactivity was not detected in the hearts of morphant zebrafish. (C) Cells positive for Isl1 are indicated with arrows. (D) There was no co-localization of HO-1 and Isl1, as indicated by the arrows. The fish is in the same orientation as that in Fig. 1.

and when exposed to hypoxia they displayed higher $\dot{Q}$ and SV. Thus, the HO-1/CO system has a negative chronotropic effect under normoxic conditions only, presumably by acting on the pacemaker cells of the heart, and, under hypoxic conditions, it regulates $\dot{Q}$ and SV.

Presence of HO-1 in the hearts of fish

Previous studies on mammals have localized HO-1 in cardiac fibroblasts, myocytes, endothelial cells and vascular smooth muscle (reviewed in Banerjee et al., 2007; Harvey and Rosenthal, 1999). However, there is no direct evidence as yet for the presence of HO-1 in the pacemaker cells of the mammalian heart. In zebrafish, the cardiac pacemaker cells are located at the sinoatrial boundary (Tessadori et al., 2012; Stoyek et al., 2015). In this study, we found evidence that HO-1 is located in the presumptive pacemaker cells of the developing zebrafish heart. This suggests indirectly that the HO-1/CO system may be involved in setting cardiac rhythm in fish. Further immunohistochemistry studies using antibodies against other pacemaker proteins such as hyperpolarization-activated and cyclic nucleotide-gated (HCN) channels coupled with Isl1 are needed to definitively state that HO-1 is indeed present in pacemaker cells of the heart. In addition, Isl1 localized to cardiac progenitors of mice (Cai et al., 2003) and thus it may also label myocyte and/or neuronal progenitors in the heart of the developing zebrafish, which explains its distribution pattern.

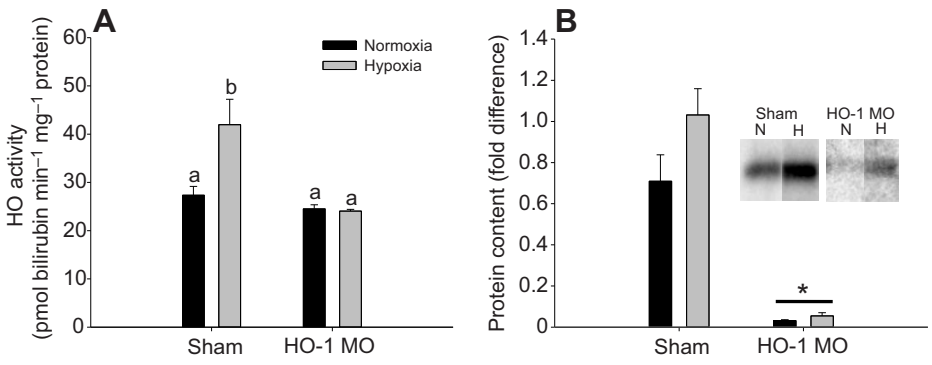

Fig. 3. HO-1 activity and protein content in 4 dpf sham-treated and HO-1 morphant zebrafish larvae. (A) HO-1 activity. (B) HO-1 protein content. Inset in B shows representative western blot bands confirming the knock down of HO-1 protein in the morphant larvae. Different letters indicate significant differences within a group. An asterisk indicates a significant difference between groups within a treatment. N, normoxia; H, hypoxia; MO, morphant. Two-way ANOVA, $P<0.05$, $N=6–8$.

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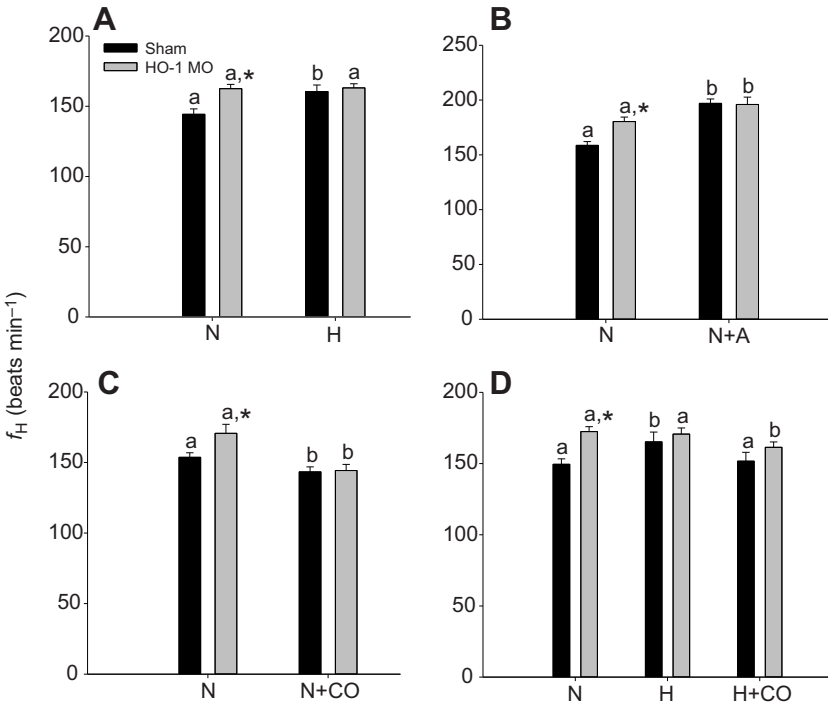

Fig. 4. Average heart rate (f_H) of 4 dpf sham-treated and HO-1 morphant zebrafish larvae. (A) Normoxia (N) and hypoxia (H). (B) Normoxia and normoxia+adrenaline (A). (C) Normoxia and normoxia+carbon monoxide (CO, 40 μmol l^{-1}, via a CO-releasing molecule). (D) Normoxia, hypoxia and hypoxia+CO. Different letters indicate significant differences between normoxia and hypoxia within either sham-treated or HO-1 morphant fish. An asterisk indicates a significant difference between sham-treated and HO-1 morphant fish within either normoxia or hypoxia. Two-way repeated measures ANOVA, P<0.05, N=8–14.

Previous studies demonstrated that exogenous application of CO to mouse sinoatrial node preparations caused an accelerated sinus rhythm, demonstrating a direct effect of CO on f_H (Abramochkin, 2013). A positive chronotropic effect of CO also was observed in the working myocardium of mouse, providing further evidence that CO is involved in controlling cardiac frequency (Abramochkin et al., 2015). The constitutively expressed form of HO, HO-2, also is present in interstitial cells of Cajal (ICC) which act as pacemaker cells in the gastrointestinal tracts of mammals (Farrugia and Szurszewski, 1999). CO synthesized from ICCs targets nearby gastrointestinal smooth muscle cells, causing the activation of a potassium current and hyperpolarization of the cell plasma membrane (Farrugia et al., 1993, 1998). However, there are no studies reporting on the presence of either isoform in the pacemaker cells of the heart.

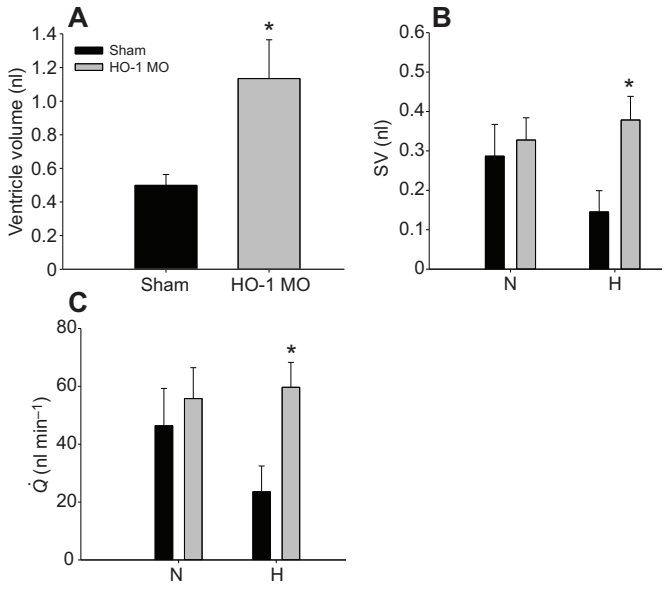

Fig. 5. The effect of heme oxygenase-1 (HO-1) on heart size, stroke volume and cardiac output of 4 dpf zebrafish larvae. (A) Ventricle volume (Student's t-test, *P<0.05). (B) Stroke volume (SV). (C) Cardiac output ($\dot{Q}$). In B and C, an asterisk indicates a significant difference between groups within an oxygen treatment (two-way repeated measures ANOVA, P<0.05, N=5–9). N, normoxia; H, hypoxia.

HO-1/CO system in control of heart function of the zebrafish larvae

The cardiotropic effects of the HO-1/CO system deserve significant attention owing to the known potency of CO as a gaseous signalling molecule or gasotransmitter. Control of f_H is particularly important under stressful conditions, such as hypoxia, when regulating cardiac rhythm is crucial for the delivery of O_2 to vital organs such as the brain. Under normoxic conditions, the developing zebrafish larva relies on diffusive cutaneous O_2 uptake and delivery of O_2 to tissues is accomplished without the requirement of hemoglobin-mediated O_2 transport (Pelster and Burggren, 1996). Indeed, because zebrafish without a beating heart develop more-or-less normally for the first few days, it is thought that internal convection (circulation) may not be required to sustain normal rates of metabolism in early life stages of zebrafish (Pelster and Burggren, 1996; Burggren, 2004; Kopp et al., 2010). However, under hypoxic conditions, the carriage of O_2 by hemoglobin is critical to sustain normal rates of O_2 uptake in zebrafish larvae (Rombough and Drader, 2009). Similarly, convective O_2 delivery to larval tissues may become more important during acute hypoxia. At such times, the ability to regulate $\dot{Q}$ via control of f_H would be highly beneficial. Our results suggest that the HO-1-CO system may be an important determinant of cardiac frequency in hypoxic larvae.

The knockdown of HO-1 caused an increase in resting f_H in normoxic larvae that was rescued by the addition of exogenous CO. These results clearly indicate a tonic inhibitory role of the HO-1/CO system on cardiac frequency in developing zebrafish. These findings are in contrast to the positive chronotropic effects reported in mammals (Abramochkin, 2013; Abramochkin et al., 2015). Based on the present study, the inhibitory effects of CO do

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Table 1. Diastolic and systolic ventricle volumes of sham-treated and heme oxygenase-1 (HO-1) morphant zebrafish exposed to hypoxia

	Ventricle volume (nl)			
	Sham-treated		HO-1 morphant	
Oxygen treatment	Diastolic	Systolic	Diastolic	Systolic
Normoxia	0.859±0.0854	0.572±0.133	1.147±0.198	0.8203±0.231*
Hypoxia	0.515±0.108‡	0.370±0.0805	1.093±0.204	0.715±0.152*

Hypoxia level, P_{O_2}=30 mmHg.
*Significant difference between diastolic and systolic volume within the same oxygen treatment for HO-1 morphant fish.
‡Significant difference from normoxic values within either diastole or systole for sham-treated fish.
Two-way ANOVA, $P<0.05$, N=5–9.

not appear to persist under hypoxic conditions because there was no further increase in f_H when HO-1-deficient larvae were exposed to hypoxia. Thus, it is possible that the prevention of CO production results in a maximal f_H under normoxic conditions and therefore leaves no scope for further increases when fish are challenged subsequently with hypoxia. Exposure to adrenaline under normoxic conditions, however, caused a significant increase of f_H in both sham-treated and HO-1-deficient larvae, indicating that larvae retain the capacity to increase f_H even when endogenous CO production is low. Similarly, inhibition of the HO/CO system in trout using the enzyme inhibitor zinc protoporphyrin-IX resulted in a significant increase in smooth muscle tension of isolated arteries but did not affect the magnitude of subsequent noradrenaline-induced contractions (Dombkowski et al., 2009). These results suggest that under normoxic conditions, at least, the effects of endogenously produced CO on the regulation of cardiovascular function are acting separately from those of catecholamines. Under hypoxic conditions, however, the HO/CO system has been shown to regulate cardiovascular function by modulating sympathetic tone in mammals (Hirakawa and Hayashida, 2006; Ding et al., 2008). In addition, it has been reported that the sympathetic nervous system is responsible for evoking the tachycardic response observed in larval zebrafish exposed to high environmental CO_2 (Miller et al., 2014) and therefore this may also be the mechanism responsible for the hypoxic tachycardia reported in this study. This notion was supported by the further increase of f_H observed in hypoxic sham-treated larvae in the presence of adrenaline (from 155.0±5.7 to 187.0±5.7 beats min^{-1}; data not shown). Furthermore, the origins of hypoxic tachycardia in zebrafish larvae may reflect an active sympathetic nervous system regulated by the HO-1/CO system under hypoxic conditions because hypoxic HO-1-deficient larval hearts were insensitive to adrenaline (from 161.0±3.4 to 181.0±4.0 beats min^{-1}; two-way repeated-measures ANOVA, P=0.091; data not shown). Thus, the inhibitory effects of CO observed under normoxia implicate an active HO-1/CO pathway in the control of resting f_H in the developing zebrafish. This tonic inhibition of cardiac activity via the HO-1/CO system may allow fish a greater capacity to increase f_H when experiencing hypoxic conditions. Moreover, the HO-1/CO system seems to be a necessary component, in coordination with the sympathetic nervous system, in regulating f_H under hypoxia because lack of CO prevented adrenaline from exerting its effects on f_H under hypoxic conditions.

Ventricle size is another indicator of heart function because it sets, in part, the volume of blood that can be pumped per heart beat (SV). In this study, we observed significantly larger ventricular volumes in HO-1-deficient larvae. Consequently, the increase in ventricular volume in HO-1-deficient larvae resulted in higher SV and $\dot{Q}$ in these fish when exposed to hypoxia. The tendency in sham-treated larvae during acute hypoxia was to increase f_H while maintaining SV and $\dot{Q}$. In sham-treated zebrafish larvae exposed to hypoxia, diastolic volume was decreased without a change in systolic volume, which, when coupled with an increase in f_H, resulted in sustained cardiac performance. Sustained cardiac performance during hypoxia also was reported in tilapia (*Oreochromis* hybrid), which compensated for a decrease in f_H induced by severe hypoxia by elevating SV and thus maintained $\dot{Q}$ (Lague et al., 2012). Based on our results, HO-1-deficient larvae were also able to maintain normoxic SV and $\dot{Q}$ under hypoxic conditions. A high f_H coupled with a larger ventricle volume may have contributed to the routine normoxic levels of SV and $\dot{Q}$ observed in HO-1-deficient larvae under hypoxic conditions. The increased ventricle size observed in HO-1-deficient larvae is in contrast to a previous report on mouse heart. Yet et al. (1999) reported that the myocardium of HO-$1^{-/-}$ mice appeared normal under normoxic conditions but under hypoxic conditions the mice developed severe right ventricular dilatation which was not observed in wild-type mice.

Possible mechanisms of HO-1/CO system in control of heart function in fish

The molecular mechanisms through which the HO/CO system exerts its effects on cardiovascular function have been well described in the mammalian literature with only a single previous study conducted on fish. Because it is so readily diffusible across membranes, CO may act quickly in either an autocrine or paracrine manner to affect cells within the vicinity of the cell in which it was synthesized. CO readily binds to heme-containing proteins and, among them, soluble guanylyl cyclase is one of the most well studied and is essential for the vasodilatory effects of CO (Wilkinson and Kemp, 2011; Abramochkin et al., 2015). In fish, the HO-1/CO pathway plays a dilatory role in blood vessels via a CO-mediated increase in cGMP (Dombkowski et al., 2009). Other CO targets may include ion channels such as voltage-gated Ca^{2+} (T-type Ca^{2+} channels; reviewed in Capel and Terrar, 2015) and K^+ channels (HCN and ERG K^+ channels) important for establishing the robust T-type Ca^{2+} current coupled with a 'funny' current (I_f) and a rapid delayed rectifier K^+ current (I_{Kr}) that are important for the diastolic depolarization rate of the developing zebrafish heart (Nemtsas et al., 2010; Alday et al., 2014). Studies from mammals have shown that endogenous CO inhibits all isoforms of T-type Ca^{2+} channels as well as ERG K^+ channels (responsible for I_{Kr}) under normoxic conditions (reviewed in Peers et al., 2015) but there is no information on the effect of endogenous CO on HCN channels (responsible for I_f). The effects of endogenous CO on the cardiac pacemaker currents has not been studied in teleost fish thus far; therefore, the following interpretation of the mechanisms of action is an extrapolation from studies on mammals. In the present study, it is possible that the HO-1/CO system acted to control resting f_H by inhibiting Ca^{2+} current via T-type Ca^{2+} channels, and subsequently reducing I_f in the pacemaker cells, resulting in a decreased f_H, which could explain the increased baseline f_H in HO-1 morphant larvae. The lack of a further increase in f_H in hypoxic HO-1 morphants may be related to the possibility that in zebrafish larvae cardiac T-type Ca^{2+} channels are activated by acute hypoxia exposure in a CO-dependent manner, in a similar fashion to L-type Ca^{2+} channels (Movafagh and Morad, 2010), and if already in their active state under normoxia because of low CO production, f_H, SV and $\dot{Q}$ remain unchanged in hypoxic HO-1 morphant larvae.

HO-1/CO system in other vertebrates

The above discussion uses the mammalian HO-1/CO system as a point of comparison for the one found in zebrafish. The HO-1/CO system, however, has been well conserved throughout all

vertebrates. The amino acid protein sequence of zebrafish (*D. rerio*) HO-1 bears an 86% identity with goldfish (*Carassius auratus*; Wang et al., 2008), 60% with *Xenopus* (Shi et al., 2008) and approximately 50% when compared with mammalian and avian protein sequences (reviewed in Olson et al., 2012). Furthermore, the amino acid sequence of the 'HO signature motif', believed to be important for heme binding and catabolism, shares 92–96% similarity between all vertebrate species investigated to date (Wang et al., 2008). Physiological control by endogenously produced CO is not restricted to mammalian vertebrates but is also involved in control of vascular tone and breathing in fish (Dombkowski et al., 2009; Tzaneva and Perry, 2014), retinal pathways and neuromuscular transmission in amphibians (Pong and Eldred, 2009; Sitdikova et al., 2007; reviewed in Sitdikova and Zefirov, 2012), and learning and vascular control in birds (Cutajar et al., 2005; Van der Sterren et al., 2011). Therefore, the HO-1/CO system appears to be a well conserved signalling pathway throughout all extant vertebrates. The reader is referred to a comprehensive review of the HO/CO system by Olson et al. (2012) for an in-depth comparison of the function of the HO/CO system in vertebrates.

Conclusions

This study implicates the HO-1/CO system in the control of cardiac function in the developing zebrafish larva. We demonstrate for the first time the presence of HO-1, and presumably the endogenous production of CO, in the pacemaker cells of the developing heart. We also show that HO-1 plays an inhibitory role in regulating resting f_H.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: V.T. and S.F.P. Acquisition of data: V.T. Analysis and interpretation of data: V.T. and S.F.P. Drafting of the manuscript: V.T. Critical revision of the manuscript for important intellectual content: S.F.P. Statistical analysis: V.T. Obtained funding: S.F.P. Administrative, technical and material support: V.T. and S.F.P. Study supervision: V.T. and S.F.P.

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Supplementary information

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