

## RESEARCH ARTICLE

# Evidence for a plasma-accessible carbonic anhydrase in the lumen of salmon heart that may enhance oxygen delivery to the myocardium

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## ABSTRACT

Oxygen supply to the heart of most teleosts, including salmonids, relies in part or in whole on oxygen-depleted venous blood. Given that plasma-accessible carbonic anhydrase (CA) in red muscle of rainbow trout has recently been shown to facilitate oxygen unloading from arterial blood under certain physiological conditions, we tested the hypothesis that plasma-accessible CA is present in the lumen of coho salmon (*Oncorhynchus kisutch*) hearts, and may therefore assist in the luminal oxygen supply to the spongy myocardium, which has no coronary circulation. We demonstrate a widespread distribution of CA throughout the heart chambers, including lumen-facing cells in the atrium, and confirm that the membrane-bound isoform *ca4* is expressed in the atrium and ventricle of the heart. Further, we confirm that CA catalytic activity is available to blood in the atrial lumen using a modified electrometric  $\Delta\text{pH}$  assay in intact atria in combination with either a membrane-impermeable CA inhibitor or specific cleavage of the Ca4 membrane anchor. Combined, these results support our hypothesis of the presence of an enhanced oxygen delivery system in the lumen of a salmonid heart, which could help support oxygen delivery when the oxygen content of venous blood becomes greatly reduced, such as after burst exercise and during environmental hypoxia.

**KEY WORDS:** Membrane-bound CA, Cardiac tissue, CA4, *Oncorhynchus*, Teleost, Root effect

## INTRODUCTION

Swimming performance in teleosts is dependent on adequate cardiac support, which in turn requires sufficient oxygen supply to the cardiac muscle. This oxygen supply is met by a variable combination of coronary circulation and cardiac circulation (i.e. venous return contained in the lumen of cardiac chambers). Very few teleost fish have a coronary supply to the entire myocardium, and the hearts of at least one-third of teleosts are completely devoid of coronary vessels (Davie and Farrell, 1991; Tota et al., 1983; Farrell et al., 2012). Oxygen diffusion from the cardiac circulation into the myocardium depends on the partial pressure gradient of oxygen in the venous

blood ( $P_{\text{VO}_2}$ ) that can decrease substantially after burst exercise (Farrell and Clutterham, 2003) and during environmental hypoxia (Holeton and Randall, 1967). Therefore, the heart appears precariously placed in the teleost circulatory design by having to derive the majority – if not all – of its oxygen supply from venous blood, and so burst exercise, environmental hypoxia and perhaps supra-optimal water temperature may be situations in which  $P_{\text{VO}_2}$  is too low to fully support the oxygen needs of the heart (Farrell, 2009). Indeed, numerous studies report threshold  $P_{\text{VO}_2}$  values below which teleost hearts fail (see Davie and Farrell, 1991). For example, a minimum  $P_{\text{VO}_2}$  of 7–10 Torr is required for Chinook salmon (*Oncorhynchus tshawytscha*) exercising at elevated temperature (Clark et al., 2008), and in rainbow trout (*O. mykiss*) exercising in hypoxic water (Steffensen and Farrell, 1998).

Given that the fish heart is poised as a potential weak link in the oxygen transport cascade, adaptations to support oxygen delivery to the myocardium should be of selective advantage. A recent theory (Randall et al., 2014) suggests that the evolutionary success of the teleost lineage owes to a series of physiological adaptations that enhance general tissue oxygen delivery, namely: (1) hemoglobins (Hbs) with high pH sensitivity; (2) intracellular red blood cell pH (RBC  $\text{pH}_i$ ) regulation; and (3) localized expression of plasma-accessible carbonic anhydrase (CA), the enzyme that catalyzes the rapid inter-conversion of  $\text{CO}_2$  and  $\text{HCO}_3^-$ . In most vertebrates, a decrease in RBC  $\text{pH}_i$  will reduce Hb- $\text{O}_2$  affinity (Bohr effect), but in some fishes, excess  $\text{H}^+$  will also reduce oxygen carrying capacity of the blood (Root effect). The Root effect is well-known for its crucial role in oxygenating the poorly vascularized retina and pressurizing gases in the swim bladder in ray-finned fishes (Berenbrink, 2011). During a general blood acidosis, such as that occurring with intense exercise or stress, the highly pH-sensitive Hb- $\text{O}_2$  binding can be a liability if Hb re-oxygenation is hindered at the gills. Thus, teleosts with highly pH-sensitive Hbs have evolved a mechanism to protect RBC  $\text{pH}_i$  whereby activation of sodium/proton exchangers (NHE), by  $\beta$ -adrenergic stimulation or otherwise, maintains RBC  $\text{pH}_i$  by extruding  $\text{H}^+$  in exchange for  $\text{Na}^+$  (Nikinmaa et al., 1990). In the absence of CA activity in the plasma,  $\text{H}^+$  extruded from the RBCs will combine with  $\text{HCO}_3^-$  to form  $\text{CO}_2$  at a slow uncatalyzed rate, enabling the RBC NHEs to maintain a pH gradient across the plasma membrane, thereby effectively protecting Hb- $\text{O}_2$  affinity. In the presence of plasma-accessible CA, however, the extruded  $\text{H}^+$  very rapidly dehydrate plasma  $\text{HCO}_3^-$ , and the resulting  $\text{CO}_2$  readily diffuses back into the RBCs where it is quickly rehydrated by intracellular CA (Rummer and Brauner, 2011).  $\text{H}^+$  production in the RBCs out-paces its extrusion by NHE because of the high catalytic activity of CA within and outside the RBCs, and the net acidification of the RBC reduces Hb- $\text{O}_2$  affinity, unloading oxygen and raising the local partial pressure of oxygen ( $P_{\text{O}_2}$ ). While experimental

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evidence in rainbow trout supports this mechanism for enhanced oxygen delivery to red muscle (Rummer et al., 2013), its involvement in oxygen delivery to other metabolically active tissues is unknown. Given the vital importance of maintaining oxygen supply to the myocardium, we tested the hypothesis that the salmon heart contains a membrane-bound CA that is accessible to blood in the lumen. We provide histological and molecular evidence of CA in the atrium and ventricle of coho salmon hearts, and demonstrate, using a novel *in vitro* assay, that membrane-bound CA is available to blood in the avascular atrial lumen. This localization of CA could facilitate oxygen delivery during critical periods of low  $P_{V_{O_2}}$  and blood acidosis, which salmon must experience during their exhausting spawning migrations.

## MATERIALS AND METHODS

### Experimental animals and heart collection

Adult female coho salmon [*O. kisutch* (Walbaum 1792)] obtained as smolts from Target Marine Hatcheries (Sechelt, BC, Canada) were housed in a flow-through outdoor 10,000 litre tank supplied with dechlorinated Vancouver city water (~12°C) at the University of British Columbia, Vancouver, BC, Canada. Fish were held under a natural photoperiod and fed every other day with commercial salmon chow. Prior to use, individual fish were terminally anesthetized in MS-222, weighed, and injected with 100 i.u. heparin into the caudal vein. The whole heart was then removed and perfused with 100 i.u. ml<sup>-1</sup> heparinized saline (in mmol l<sup>-1</sup>: 124.1 NaCl, 2.50 KCl, 0.93 MgSO<sub>4</sub>·7 H<sub>2</sub>O, 2.52 CaCl<sub>2</sub>·2 H<sub>2</sub>O, 5.55 glucose, 3.87 TES acid and 6.13 TES salt, pH 7.4) to remove blood from the lumen prior to further processing, as described below. Care and use of the animals was approved by the University of British Columbia Animal Care Committee according to the principles of the Canadian Council for Animal Care.

### Enzyme histochemistry

Following saline perfusion, hearts were immersed in phosphate buffered saline (PBS; pH 7.4) containing 4% paraformaldehyde and 1.5% glutaraldehyde for 4 h at room temperature, then cryoprotected in a 30% sucrose solution in PBS prior to embedding in Cryomatrix (Thermo Shandon, Pittsburgh, PA, USA). Cryosections (16 µm) were thaw-mounted onto filter paper discs (pore size 0.45 µm; Millipore, Bedford, MA, USA), air-dried, then processed immediately or temporarily stored at -20°C. CA enzymatic activity was confirmed using Hansson's histochemical method of cobalt sulphide precipitation (Hansson, 1967). Briefly, individual filter paper discs containing heart sections were floated on the surface of a small weigh boat filled with freshly prepared reaction solution (in mmol l<sup>-1</sup>: 1.75 CoSO<sub>4</sub>, 53 H<sub>2</sub>SO<sub>4</sub>, 11.7 KH<sub>2</sub>PO<sub>4</sub>, 15.7 NaHCO<sub>3</sub>; with or without 10<sup>-5</sup> mol l<sup>-1</sup> acetazolamide) for 30 min at room temperature. After rinsing briefly in 0.67 mmol l<sup>-1</sup> phosphate buffer (pH 5.9), sections were immersed in 0.5% (NH<sub>4</sub>)<sub>2</sub>S in MilliQ water for 3 min and then rinsed several times in water. To ensure that the integrity of the tissue slice was maintained for imaging, filter papers were mounted directly onto glass slides and then photomicrographed with a 20× objective on a Nikon Eclipse 90i.

### Cloning and expression of *ca4* in the heart

Freshly dissected tissues (gill, bulbus arteriosus, atrium, ventricle, and separated spongy and compact layers of ventricle) were placed in RNAlater, and then total RNA was extracted using Trizol (Life Technologies, Carlsbad, CA, USA). Following DNase I treatment, 500 ng RNA was reverse transcribed to cDNA using the High Capacity Reverse Transcription Kit following the manufacturer's

instructions throughout (Life Technologies). Duplicate cDNA reactions lacking the Multiscribe RT enzyme were included for each sample to control for genomic DNA contamination. Degenerate primers were designed for *ca4* (forward: 5'-CAGTC-CCCATYAAACATTGT-3', reverse: 5'-GAGCCMTGRTAGCGG-WAGTA-3') based on a homology alignment of *O. mykiss* and *Squalus acanthias* (accession numbers AY514871 and DQ092628, respectively), and used in standard polymerase chain reactions on atrium cDNA with OneTaq DNA Polymerase (New England Biolabs, Whitby, ON, Canada). A single product of the expected size was purified, subcloned (PGEM-T Easy Vector and JM109 competent cells, Promega, Madison, WI, USA) and sequence-verified. RT-PCR was repeated as above on cDNAs from all isolated tissues using nested primers specific to this sequence (forward: 5'-GATTGGTGGAGACCTGGAGA-3'; reverse: 5'-GTCTTTCAG-GGCATCTTCCA-3'). Cytoplasmic  $\beta$ -actin was amplified in separate reactions for quality control (primers from Veldhoen et al., 2010). All non-RT control templates failed to amplify.

### CA assays in atrial chambers

The atrium was isolated intact and fitted with afferent and efferent cannulae (flared PE60 tubing secured with 4-0 silk sutures). The afferent cannula was modified to include a short nested cannula of PE10 tubing glued in place. In Experiment 1, cannulated atria ( $n=6$ ) were connected to a stop-flow apparatus that allowed the atrial chamber to be continuously perfused at 1 ml min<sup>-1</sup> with assay buffer (in mmol l<sup>-1</sup>: 225 mannitol, 75 sucrose, 10 Tris base, adjusted to pH 7.4 at 4°C with 10% phosphoric acid; Henry, 1991) from an aerated, constant-pressure reservoir maintained at 4°C. A needle-type pH microsensor (PreSense, Regensburg, Germany) was inserted through the myocardium and positioned in the approximate center of the atrial lumen. Chamber pH was continuously recorded to the nearest 0.01 pH units in real time at a frequency of once per second (pH1 View, Presense) while assay buffer continued to be perfused. CA activity in the atrial lumen was measured using the electrometric  $\Delta$ pH method (Henry, 1991), where perfusion was stopped and the CO<sub>2</sub> hydration reaction was initiated by adding 20–40 µl (~10% luminal volume) CO<sub>2</sub>-saturated MilliQ water through the nested cannula. After the reaction had proceeded to completion (stable pH), perfusion was re-initiated and the procedure was repeated twice. Next, the chamber was perfused with assay buffer containing 200 µmol l<sup>-1</sup> of C18, a membrane impermeable CA inhibitor (Supuran et al., 2000; Rummer et al., 2013), and the CO<sub>2</sub> hydration rate was measured in triplicate as above. Finally, the chamber was perfused again with assay buffer in the absence of C18 and the CO<sub>2</sub> hydration rate was re-measured as above to confirm that the inhibition was reversible. Atria were then blotted dry and weighed.

In Experiment 2, atria were isolated and cannulated as above, and then filled with 400 µl of either saline alone or saline containing 1 i.u. phosphatidylinositol-specific phospholipase C (PI-PLC; Life Technologies) and incubated at room temperature for 90 min ( $n=6$ ). PI-PLC is known to release glycoposphatidylinositol (GPI)-anchored proteins, such as Ca<sub>v</sub>4, from cell surfaces (Cross, 1987; Low et al., 1988; Zhu and Sly, 1990; Bottcher et al., 1994; Gilmour et al., 2002; Esbaugh and Tufts, 2004). Immediately following this pre-treatment, individual atria were perfused with assay buffer and the CO<sub>2</sub> hydration rate was measured in triplicate as described above. Atria were blotted dry after the assay and weighed.

### Data analysis

Initial pH (pH<sub>1</sub>) was valued as the average pH recording for the 30 s after the flow was stopped and prior to CO<sub>2</sub> injection, and final pH

( $pH_2$ ) was determined as the average pH recording for the final 15 s of the assay prior to re-initiating flow. Triplicate recordings were analyzed to determine the average reaction velocity ( $V$ , in pH units  $s^{-1}$ ) for each atrial chamber as follows:

$$V = \Delta pH / \Delta t, \quad (1)$$

where  $\Delta pH = pH_2 - pH_1$ , and  $\Delta t$  is the time (s) between the  $pH_1$  and  $pH_2$  measurements. Despite large differences in  $\Delta t$  between treatments for both experiments,  $\Delta pH$  varied on average less than 0.05 pH units with intra-assay coefficients of variation of 36.9% and 25.9% for Experiments 1 and 2, respectively. For Experiment 1, a one-way repeated-measures ANOVA (RM ANOVA) and Holm–Sidak *post hoc* test for multiple comparisons were used to determine differences in  $V$  between control, C18 inhibited and recovery traces ( $n=6$ ,  $P<0.05$ ). For Experiment 2, a Student's two-tailed *t*-test was used to compare the  $V$  of control and PI-PLC-treated atria ( $n=6$ ,  $P<0.05$ ). A one-way ANOVA was used to compare atrial mass among assay groups. All data are expressed as means  $\pm$  s.e.m.

## RESULTS

### Distribution of CA in the heart

CA activity and distribution in the coho heart was visualized by enzyme histochemistry using cobalt sulphide precipitation (Hansson, 1967). In this technique, cobalt precipitates at sites of CA activity as the enzyme catalyzes the alkalization of the reaction solution (Maren, 1980), and the black deposits are readily visualized in tissue sections. Multiple cell types were stained with this technique, as distinguished

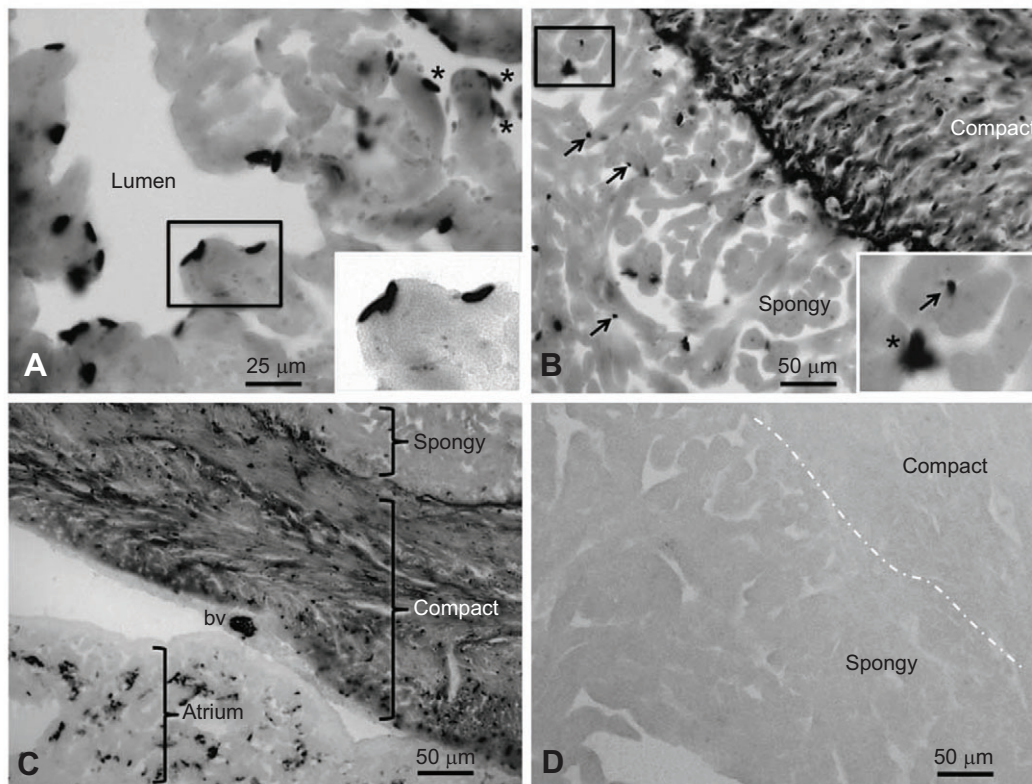
by size, shape and location (Fig. 1). In the atrium, squamous endothelial cells lining the lumen were frequently seen (Fig. 1A). In the ventricle, myocytes in the compact layer were abundantly stained and clearly demarcated the spongy layer boundary (Fig. 1B). Small round cells in the spongy layer of the ventricle (Fig. 1B) were readily distinguished from the few large, plump and deeply stained RBCs scattered in the lumens of the heart and blood vessels (Fig. 1A–C). No staining was observed in the bulbus arteriosus (not shown). The specificity of the staining protocol was confirmed by inhibiting CA activity with the addition of acetazolamide to the reaction solution, which completely abolished cobalt precipitation (Fig. 1D).

### Cloning and expression of *ca4*

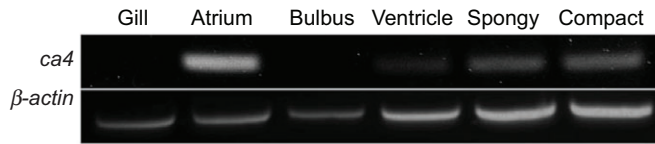
Homology cloning using atrium cDNA yielded a 472 bp fragment of a CA sequence that shared 69–83% identity with other known teleost *ca4* sequences (GenBank accession number KT362379). The coho *ca4* transcript was expressed in all of the contractile regions of the heart (atrium, ventricle, isolated spongy and compact layers of the ventricle) but was not detected in the bulbus arteriosus or gills (Fig. 2).

### CA activity in the atrial lumen

To functionally demonstrate that CA localized in endocardial cells is oriented towards the plasma, we modified the electrometric  $\Delta pH$  assay (Henry, 1991) to measure CA activity in the lumen of intact atria. The atrium was chosen for this assay for two reasons: (1) it is avascular and therefore wholly dependent on luminal oxygen



**Fig. 1. Distribution of carbonic anhydrase (CA) in the coho heart.** CA activity in 16  $\mu m$  transverse cryosections of the coho heart was visualized by enzyme histochemistry using cobalt sulphide precipitation (Hansson, 1967). (A) Staining in the atrium predominated in squamous endocardial-type cells (inset) lining the lumen, and in red blood cells (\*) scattered throughout the lumen. (B) The compact layer of the ventricle was more heavily stained compared with the spongy layer. The inset in B contrasts a small round ventricular cell (arrow) with a cluster of two to three red blood cells (\*) in the spongy ventricle. (C) Comparison of overall staining patterns in the atrium and ventricle, including a small blood vessel (bv) containing red blood cells in the epicardium. (D) Specificity of staining was confirmed with the addition of  $10^{-5} \text{ mol l}^{-1}$  acetazolamide to the reaction solution, which completely abolished staining. The white dot-dashed line indicates the boundary between the ventricular compact and spongy layers, which is deeply stained in B.



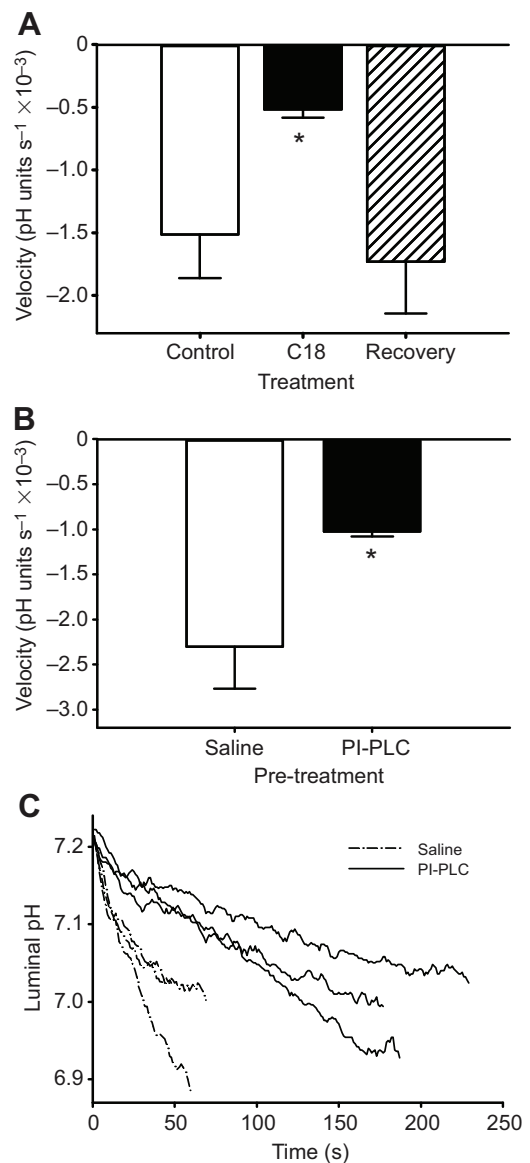
**Fig. 2. Expression of carbonic anhydrase 4 (*ca4*).** The regional expression of *ca4* in the heart was compared by RT-PCR, with gill tissue included as a negative control (Georgalis et al., 2006) and  $\beta$ -actin as a positive control. The cardiac regions isolated were: atrium, bulbus arteriosus, whole ventricle, and separated spongy layer of the ventricle and isolated compact layer of the ventricle. All non-reverse-transcribed templates failed to amplify products of either gene (not shown).

supply, and (2) the relatively larger atrial lumen that is free of trabeculae, as compared with the ventricle, allowed consistent placement of the delicate pH microsensor into its lumen. In Experiment 1, individual atria were assayed sequentially with and without the membrane-impermeable CA inhibitor C18. In all atria, inclusion of C18 in the reaction buffer reduced the reaction velocity threefold from the initial uninhibited control reaction ( $-5.2 \times 10^{-4} \pm 6.6 \times 10^{-5}$  versus  $-1.5 \times 10^{-3} \pm 3.5 \times 10^{-4}$  pH units  $s^{-1}$ , respectively;  $n=6$ ,  $P<0.001$ ; Fig. 3A). This inhibition by C18 of CA in the atrial chambers was reversible, with reaction velocities returning to control values upon removal of the inhibitor ( $128.7 \pm 35.7\%$  recovery;  $P>0.05$ ; Fig. 3A). As further confirmation for the orientation of CA on atrial endothelial cells, and to help elucidate which CA isozymes may be present, Experiment 2 began by pre-incubating the cannulated atria with saline either alone or in combination with PI-PLC. PI-PLC is an enzyme that specifically cleaves the GPI membrane anchor of CA4 in a broad range of organisms, including fish (Esbaugh and Tufts, 2004; Gilmour et al., 2002). The PI-PLC-treated atria had an average reaction velocity of  $-1.0 \times 10^{-3} \pm 5.6 \times 10^{-5}$  pH units  $s^{-1}$ , which was over twofold slower than the reaction velocity of control atria pre-treated with saline alone ( $-2.3 \times 10^{-3} \pm 4.6 \times 10^{-4}$  pH units  $s^{-1}$ ;  $n=6$ ;  $P=0.02$ ; Fig. 3B). In both Experiments 1 and 2, the triplicate assays yielded highly reproducible traces of the decrease in pH associated with  $CO_2$  hydration, although the rate of acidification varied with treatment (Fig. 3C). There was no significant difference in atrial mass between assays (Experiment 1:  $84.9 \pm 2.5$  mg; Experiment 2: control  $83.8 \pm 13.9$  mg, PI-PLC  $68.8 \pm 7.8$  mg;  $P=0.42$ ).

## DISCUSSION

The results of this study show that CA, including the membrane-bound isoform *ca4*, is broadly expressed in the salmonid heart and that either inhibiting or removing CA from the blood-facing membrane of endocardial cells within the atrial lumen slowed the  $CO_2$  hydration rate. This study, therefore, is the first to demonstrate a functional plasma-accessible and membrane-bound CA in the salmonid heart lumen. Given that plasma-accessible CA can facilitate oxygen unloading from the highly pH-sensitive teleost Hb during a blood acidosis (Rummer and Brauner, 2015, 2011), our findings support the hypothesis of an enhanced oxygen delivery system in the salmonid heart, one that is similar to that described in rainbow trout red muscle (Rummer et al., 2013). Such a system could become critical for this vital organ during periods of low  $P_{V_{O_2}}$  because much of the myocardium is dependent on oxygen diffusion from the venous return in the lumen. But as discussed below, there could also be other functional advantages to having plasma-accessible CA in the heart lumen.

We demonstrated the widespread distribution of CA in the coho heart by using Hansson's enzyme histochemical method (Hansson,



**Fig. 3.  $CO_2$  hydration rates within atrial chambers of coho salmon.**

Carbonic anhydrase (CA) activity was measured within atrial chambers by quantifying the velocity of  $CO_2$  hydration (pH units  $s^{-1}$ ). (A)  $CO_2$  hydration rates in atria perfused sequentially with assay buffer alone (control; white bar), buffer containing a membrane-impermeable CA inhibitor (C18; black bar) and again with assay buffer alone (recovery; hatched bar). Graph depicts the mean  $\pm$  s.e.m. reaction velocities for six atria. (B) In a separate experiment, atrial chambers were pre-treated with saline (white bar) or phosphatidylinositol-specific phospholipase C (PI-PLC; black bar) to cleave the membrane anchor of CA, then  $CO_2$  hydration rate was measured in triplicate for each atrium (mean  $\pm$  s.e.m.;  $n=6$ ). (C) Representative replicate traces from two atrial chambers, one pre-treated with saline (dashed lines) and the other with PI-PLC (solid lines). Asterisks in A and B indicate significant differences between treatments ( $P<0.05$ ).

1967), which relies on the precipitation of cobalt to visualize sites of CA activity in the tissue sections (Maren, 1980). The multiple cell types and unique staining patterns of the different heart regions observed using this technique suggests an array of functions for CA in the heart. Important to the present study, numerous squamous cells lining the lumen of the atrium were stained, supporting CA activity in the endocardium of the heart. To determine whether the CA expressed in the atrium included a membrane-bound isoform,

we cloned a *ca4* fragment from the coho atrium and demonstrated its expression in the contractile regions of the heart. This extends earlier findings of Ca4 protein expression in rainbow trout hearts and absence in gills (Georgalis et al., 2006), and of membrane-associated CA in the hearts of cyclostomes (Esbaugh and Tufts, 2004; Esbaugh et al., 2009) that are completely avascular (Farrell et al., 2012).

There are 16 CA isozymes characterized in mammalian systems and many have also been identified in other vertebrate species. The CA4 isozyme has a high catalytic activity and is anchored to the plasma membrane by a GPI residue (Esbaugh and Tufts, 2006; Hilvo et al., 2005). In mammalian hearts, CA4 is localized to the endothelium of blood vessels, the sarcolemma and the sarcoplasmic reticulum (Sender et al., 1998), and CA4 functions in facilitating the efflux of metabolic wastes into the blood and in  $\text{Ca}^{2+}$  cycling during muscle contraction and relaxation (Geers and Gros, 2000). Similarly, in teleosts, CA in skeletal muscle is also important for facilitating the diffusion of metabolic wastes into the blood. Perfusion of isolated rainbow trout trunk muscle with various CA inhibitors demonstrated a role for both intra- and extracellular CA in  $\text{CO}_2$  and  $\text{NH}_3$  excretion, although the relative contribution of these CAs varies depending on the extent of  $\text{CO}_2$  production in the muscle (Henry et al., 1997; Wang et al., 1998). More recently, plasma-accessible CA in rainbow trout red muscle was shown to enhance tissue oxygen delivery during a hypercarbia-induced blood acidosis (Rummer et al., 2013), and we propose that the *ca4* expressed in the coho heart may also enhance oxygen delivery to the myocardium in a similar manner. Although plasma-accessible CA surely plays additional functional roles in the heart, including associated gas exchange and metabolic waste removal, we focus most of the following discussion on how this localization may enhance oxygen delivery.

The model for enhanced tissue oxygen delivery in teleosts requires localized catalytic activity of CA in the plasma to short-circuit RBC pH; regulation during an acidosis in order to acidify the RBCs and reduce Hb– $\text{O}_2$  affinity (Randall et al., 2014). Thus, CA would need to be located on the luminal wall of the coho atrium to participate in enhancing oxygen delivery to the myocardium. To functionally demonstrate this distribution, we measured CA activity in the lumen of intact atria using a modification of the electrometric  $\Delta\text{pH}$  assay that quantifies the rate of  $\text{CO}_2$  hydration. Repeating the assay in the same atria in the presence of the membrane-impermeable CA inhibitor C18 caused a reversible reduction in the measured reaction velocity. Similarly, pretreating the atria with PI-PLC to cleave the CA membrane anchor also significantly decreased the reaction rate compared with saline-treated control atria. Together, these results confirm the presence of a plasma-accessible CA in the salmonid atrium that is likely the GPI-linked membrane-bound isozyme Ca4. Given that plasma-accessible CA in rainbow trout can more than double tissue  $P_{\text{O}_2}$  under relevant physiological conditions (Rummer and Brauner, 2015; Rummer et al., 2013), our demonstration of a similar CA distribution in the heart of a closely related species may have significant bearing on our current understanding of myocardial oxygenation in salmonids and other teleosts.

The modified electrometric  $\Delta\text{pH}$  assay used in this study, in combination with the CA inhibitor C18, provides functional evidence for the presence and luminal orientation of membrane-bound CA. These insights cannot be obtained by studying isolated plasma membranes because of the high CA activity in the microsomal fraction (Bruns and Gros, 1992; Geers et al., 1992), as CA4 is present on both endothelial cell and cardiomyocyte membranes (Knüppel-Ruppert et al., 2000; Sender et al., 1998), as

well as potential contamination from intracellular CA4 activity (Schneider et al., 2013). Nonetheless, limitations to our method include the inability to measure the true uncatalyzed reaction rate and a lack of mixing of the reaction volume, which prevent quantification of absolute CA activity.

We have now added a novel discovery to illustrate how well the salmonid heart is adapted for  $\text{O}_2$  uptake despite its seemingly precarious dependence on the  $P_{\text{VO}_2}$  of the  $\text{O}_2$ -depleted venous blood. In addition to plasma-accessible CA on the luminal wall that could drive up the local  $P_{\text{O}_2}$  gradient, other adaptations include the high surface area of the spongy ventricular myocardium, the arrangement of trabeculae into thin sheets of muscle to reduce the diffusion distance (Pieperhoff et al., 2009), the narrow spindle-shaped myocytes that minimize intracellular diffusion distances, and a high capillary density in the compact ventricular myocardium (Egginton and Cordiner, 1997; G. K. Cox, personal communication). Under routine conditions, this morphology is suggested to be more than sufficient for meeting myocardial oxygen demand (Farrell, 2002). Similarly, these morphological characteristics would aid the diffusion of metabolically produced  $\text{CO}_2$  from cardiomyocytes, a process that is facilitated by plasma-accessible CA in striated muscle capillaries of fish (Henry et al., 1997) and other vertebrates (Geers and Gros, 2000). Plasma-accessible CA may become critical for cardiac function during stressful situations in which normal  $P_{\text{VO}_2}$  might be insufficient to sustain cardiac function, such as during high levels of aerobic exercise, recovery from burst exercise, environmental hypoxia and supra-optimal temperature. All of these situations are stressful to the animal, can lower blood pH as a result of metabolic or respiratory acidosis, and will trigger catecholamine release into the venous blood. They also raise  $\text{CO}_2$  and other waste production by the heart, which may be working harder. Circulating catecholamines will activate RBC  $\beta\text{NHEs}$  and create a pH disequilibrium across the RBC membrane, which will be short-circuited in the presence of plasma-accessible CA, potentially enhancing  $\text{O}_2$  unloading to the cardiac tissue (Rummer and Brauner, 2011). Therefore, the physiological implications associated with the enhanced myocardial oxygenation offered by plasma-accessible CA in the heart are considerable. Future challenges will include quantifying the contribution of CA-mediated  $\text{O}_2$  delivery to the myocardium under such stressful conditions and applying this information to existing knowledge of cardiac function. Of further interest, individual variation in heart CA activity could offer a powerful scaffold for selective pressures to act upon and may contribute to observed fitness differences within populations (Eliason et al., 2011, 2013). In the present study, variation in reaction velocities of either C18-inhibited or PI-PLC-treated atria were considerably lower than those of the control reactions (s.e.m. 5–12% versus 20–24%, respectively), which is consistent with reduced variability in the treatment groups that is due to substantial inhibition/removal of CA. The higher variability in the control groups may reflect inter-individual differences in luminal CA abundance. Given the potential role of this enzyme in  $\text{O}_2$  delivery to the heart, this could dictate which fish arrive at the spawning grounds and which perish on the way, failing to reproduce.

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#### Competing interests

The authors declare no competing or financial interests.

## Author contributions

S.L.A., T.S.H., A.P.F. and C.J.B. devised the study. S.L.A. and T.S.H. conducted the experiments. J.M.W. assisted with the enzyme histochemistry. C.T.S. provided the C18. S.L.A. analyzed the data and wrote the manuscript, with intellectual and editorial contributions by all authors throughout. All authors approved the manuscript.

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