Comparative Biochemistry and Physiology, Part A 277 (2023) 111368

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

journal homepage: www.elsevier.com/locate/cbpa

Research Article

Functional divergence of teleost carbonic anhydrase 4

Angelina M. Dichiera [a,*], Valerie De Anda [b], Kathleen M. Gilmour [d], Brett J. Baker [b,c], Andrew J. Esbaugh [b]

[a] Department of Zoology, The University of British Columbia, Vancouver, BC V6T 1Z4, Canada
[b] Marine Science Institute, The University of Texas at Austin, Port Aransas, TX 78373, USA
[c] Department of Integrative Biology, The University of Texas at Austin, Austin, TX 78712, USA
[d] Department of Biology, University of Ottawa, Ottawa, ON K1N 6N5, Canada

ARTICLE INFO

Edited by Michael Hedrick

Keywords:
Tissue oxygen delivery
Acid-base balance
Ion regulation
Evolution

ABSTRACT

The functional role of membrane-bound carbonic anhydrases (CAs) has been of keen interest in the past decade, and in particular, studies have linked CA in red muscle, heart, and eye to enhanced tissue oxygen extraction in bony fishes (teleosts). However, the number of purported membrane-bound CA isoforms in teleosts, combined with the imperfect system of CA isoform nomenclature, present roadblocks for ascribing physiological functions to particular CA isoforms across different teleost lineages. Here we developed an organizational framework for membrane-bound CAs in teleosts, providing the latest phylogenetic analysis of extant CA4 and CA4-like isoforms. Our data confirm that there are three distinct isoforms of CA4 (a, b, and c) that are conserved across major teleost lineages, with the exception of CA4c gene being lost in salmonids. Tissue distribution analyses suggest CA4a functions in oxygen delivery across teleost lineages, while CA4b may be specialized for renal acid-base balance and ion regulation. This work provides an important foundation for researchers to elucidate the functional significance of CA4 isoforms in fishes.

1. Introduction

Carbonic anhydrase (CA) is a metalloenzyme found in all three domains of life (Supuran, 2008), and is responsible for catalyzing the reversible hydration/dehydration reaction of carbon dioxide (CO_2). The original gene family likely arose more than half a billion years ago prior to the division of prokaryotes and eukaryotes, and underwent several rounds of duplication that resulted in the three families (α, β, γ) and dozens of genes recognized today (Banerjee and Deshpande, 2016; Supuran, 2008). All vertebrate CA isoforms are part of the α-CA family and generally have a conserved catalytic mechanism (Banerjee and Deshpande, 2016), and yet contribute to a diverse range of physiological roles including respiration, ion regulation, and metabolism, among others (Tashian, 1989). There are 16 known isoforms from the α-CA family found in mammals, and these can be divided into distinct groups: cytoplasmic isoforms (1, 2, 3, 7, and 13), mitochondrial isoforms (5a and 5b), a secreted isoform (6), membrane-associated isoforms (4, 9, 12, 14, and 15), and the CA-related proteins (8, 10, and 11). The membrane-associated isoforms can be further divided into transmembrane and glycosylphosphatidylinositol (GPI)-linked isoforms. CA9, CA12, and CA14 are bound to membranes via the presence of transmembrane domains whereas CA4 and CA15 are bound by the presence of a GPI anchor.

Unfortunately, the mammalian-centric nomenclature used to delineate CA isoforms and subsequently ascribe physiological function is not always appropriate for non-mammalian species (Esbaugh et al., 2005; Tufts et al., 2003). Owing to additional whole genome duplication (WGD) events that occurred for fishes, there exist fish CA isoforms for which there is no mammalian counterpart. Whereas the vertebrate lineage underwent two WGDs, a third teleost-specific WGD occurred ~350 million years ago, and a fourth salmonid-specific WGD occurred more recently, ~96 million years ago (Berthelot et al., 2014). These duplications have resulted in what appears to be genetic divergence within the teleost CA gene family, where multiple isoforms perform separate functional roles. For example, teleosts display two distinct CA2-like isoforms in red blood cells (RBCs) (Esbaugh et al., 2004; Esbaugh et al., 2005; Lin et al., 2008). Both of these CA2-like isoforms demonstrate high activity levels, but CA17b (also described as 2b, 2-like b, CA-b) is found only in high abundance in the RBCs and thought to be key for CO_2 excretion. Conversely, teleost CA17a (2-like a, CA-c) is expressed in

* Corresponding author.
E-mail address: dichiera@zoology.ubc.ca (A.M. Dichiera).

https://doi.org/10.1016/j.cbpa.2023.111368
Received 14 October 2022; Received in revised form 14 December 2022; Accepted 10 January 2023
Available online 13 January 2023

multiple tissues but has little or no expression in the RBCs and is thought to play roles in acid-base balance and ion regulation (Esbaugh et al., 2004; Esbaugh et al., 2005; Lin et al., 2008). This issue has now been relatively well-resolved for cytoplasmic isoforms (Esbaugh et al., 2004; Esbaugh et al., 2005; Esbaugh and Tufts, 2006; Ferreira-Martins et al., 2016; Gilmour, 2010; Gilmour and Perry, 2009; Lin et al., 2008; Santovito et al., 2012), but the diversity and organization of membrane-associated CA isoforms in fishes, particularly the GPI-linked CA isoforms, remains unclear.

Lin et al. (2008) first presented the idea that fishes may exhibit functional specialization of CA4 isoforms by describing the presence of multiple GPI-anchored membrane-bound CA isoforms in the freshwater zebrafish (*Danio rerio*) genome. While mammals express only one CA4 isoform, nine membrane-bound isoforms were identified for zebrafish, with three CA clades each expressing a, b, and c isoforms (CA4a-c, CA15a-c, and CA16a-c) (Lin et al., 2008). Lin et al. (2008) described zebrafish CA4a-c isoforms as closely related to mammalian CA4 and other teleost CA4s, whereas novel zebrafish CA15 and CA16 isoforms (described as "CA4-like" isoforms) grouped more closely with each other (Gilmour and Perry, 2009; Lin et al., 2008). Since the classification and physiological characterization of these teleost membrane-bound CAs over a decade ago, the genomic era has ushered in dramatic increases in the number of "CA4-like" sequences derived from whole genome sequencing and transcriptomic studies that have become publicly available. Despite this, little is known about the phylogenetic relationships that define the divergence of CA4 isoforms in fishes, and the functional roles of CA4 and CA4-like isoforms across bony fish lineages.

In particular, the role of GPI-linked CA4s in teleost respiratory physiology has been of keen interest in the past decade, during which studies have linked CA4 in the red muscle, heart, and eye to enhanced tissue oxygen (O_2) extraction (Alderman et al., 2016; Brauner and Harter, 2017; Damsgaard et al., 2020; Dichiera and Esbaugh, 2020; Harter et al., 2019; Harter and Brauner, 2017; Randall et al., 2014; Rummer and Brauner, 2011; Rummer and Brauner, 2015; Rummer et al., 2013). Beyond O_2 delivery, CA4s and CA4-like isoforms have been implicated in a number of other physiological processes: pH balance during capillary transit (Henry and Swenson, 2000), bicarbonate resorption in the kidney lumen (Georgalis et al., 2006; Pelis et al., 2003), and acid excretion in the swim bladder and intestine (Georgalis et al., 2006; Gervais and Tufts, 1998; Grosell et al., 2007; Grosell et al., 2009b; Pelster, 1995; Würtz et al., 1999). Notably, however, it does not appear that CA4 contributes to CO_2 excretion at the gill of teleost fish, as it does in elasmobranchs (Gilmour and Perry, 2010; McMillan et al., 2019). The initial discovery of multiple CA4 and CA4-like proteins suggested that these functional roles might be performed by separate isoforms (Lin et al., 2008). For example, zebrafish CA4a was highly expressed in the muscle, eye, and brain, suggesting a function in tissue O_2 delivery, whereas the "CA4-like" CA15a was highly expressed in the gill and demonstrated a role in acid-base and ion regulation in larval zebrafish (Lin et al., 2008). Yet no research has expanded this concept of functional divergence across membrane-bound CAs to other teleosts, which comprise the most diverse vertebrate group on the planet.

With the diverse physiological functions described for CA4 and the plethora of publicly available CA4-like sequences, there is a clear need to identify the phylogenetic relationships and functional roles of CA4-like isoforms. On this background, the goal of the present study was to integrate both publicly available genomic data and gene expression patterns to understand the functional roles of CA4 and CA4-like isoforms in teleosts. Here, we defined the phylogenetic relationships among extant CA4 and CA4-like isoforms across a diverse array of teleost lineages in order to provide an up-to-date view of the distribution and abundance of isoforms. We then used tissue-specific mRNA expression to infer the functional significance of CA4 isoforms across teleost species using representatives of three different subcohorts. With this data, we provide an organizational framework for CA4 and CA4-like isoforms in teleosts as a guide to identify target proteins for functional studies.

2. Methods

2.1. Phylogenetic analysis of membrane-bound carbonic anhydrase

Homologues of CA4 and CA15 were retrieved from NCBI using the following search terms: "carbonic anhydrase 4 AND bony fish", "carbonic anhydrase IV AND bony fish", "carbonic anhydrase 15 AND bony fish", and "carbonic anhydrase XV AND bony fish". Since many sequences are also labeled as "-like", we expanded the search terms to include: "carbonic anhydrase 4-like AND bony fish", "carbonic anhydrase IV-like AND bony fish", "carbonic anhydrase 15-like AND bony fish", and "carbonic anhydrase XV-like AND bony fish". We substituted "bony fish" with "cartilaginous fish" in the previous search terms to retrieve sequences to use as an outgroup in the phylogenetic analysis. While Lin et al. (2008) included zebrafish CA16s in their analysis of zebrafish membrane-bound CA isoforms, these original hypothetical isoforms (zCA16a, b, and c) are now designated as either CA15 or CA-related proteins. Therefore, we did not include CA16 in our search terms. After manual refinement, removing duplicates and short partial sequences, a total of 438 non-redundant homologues of CA4 and CA15 nucleotide sequences were aligned using MAFFT v7.450 (algorithm autoselection) (Katoh and Standley, 2013) with a BLOSUM62 scoring and redefined with MUSCLE v3.8.425 (default parameters). Gaps present in at least 50% of the taxa were masked from the final alignment using Geneious Prime 2020.0.5. The phylogenetic tree was generated using IQ-tree v1.6.1 with a best-fit TVM + F + R7 model selected using the Bayesian Information Criterion (BIC). The tree was rooted using the chondrichthyan fish *Callorhinchus milii* (elephant shark) CA4 (AFP05153.1).

Taxonomy was obtained for each of the CA4 and CA15 homologues sequences that was used for phylogenetic analysis by mining FishBase using the package 'rfishbase' in R (Version 4.0.2). Fish were further classified and grouped according to the phylogenetic classification of bony fish by Betancur-R et al. (2017) and Hughes et al. (2018). Taxonomic levels (e.g. cohort, subcohort) were mapped onto the phylogenetic tree using iTOLv5 (Letunic and Bork, 2019). To further understand the relatedness of the CA4 isoforms targeted for tissue distribution below, the coding regions of the amino acid sequences for zebrafish (*Danio rerio*), rainbow trout (*Oncorhynchus mykiss*), and red drum (*Sciaenops ocellatus*) CA4s were examined for percent identity using EMBL-EBI Clustal Omega Multiple Sequence Alignment (Madeira et al., 2022). These sequences were also aligned with and compared to human CA4 (NP_000708.1).

2.2. mRNA tissue distribution for representative fish species

Sexually mature zebrafish (pet store strain) were obtained from a local pet store (Corpus Christi, TX) and held in 20 l tanks with dechloraminated Port Aransas, TX tap water at ambient room temperature (22 °C). Red drum were obtained from Ekstom Aquaculture, LLC in El Campo, TX and held in 600 l tanks receiving full-strength recirculating seawater (35 ppt; 22 ± 2 °C). Tissues were collected from zebrafish and red drum after euthanasia via immersion in tricaine methanesulfonate (MS-222; 250 mg l^{-1} for euthanasia, buffered with 500 mg l^{-1} $NaHCO_3$), snap frozen, and stored at −80 °C until use. Juvenile rainbow trout were purchased from Linwood Acres Trout Farm (Campbellcroft, ON, Canada) and transported to the University of Ottawa. Trout were held in 1275 l fiberglass holding tanks containing aerated, dechloraminated municipal tap water at 13 °C. Tissues were collected from trout after euthanasia via immersion in an aerated benzocaine solution (50 mg l^{-1} ethyl p-aminobenzoate; Sigma Aldrich). As above, samples were snap frozen and stored at −80 °C until they were shipped to the University of Texas Marine Science Institute on dry ice. Upon arrival, samples were stored at −80 °C until processed. Zebrafish and red drum were handled according to the University of Texas at Austin Institutional Animal Care and Use Committee guidelines (protocol AUP-2018-00231), while

rainbow trout were handled under the auspices of the University of Ottawa institutional Animal Care Committee and in compliance with the guidelines of the Canadian Council on Animal Care (protocol BL-2118).

Red drum ($n = 4$), rainbow trout ($n = 7$) and zebrafish ($n = 16$) were sampled for the following tissues: gill, heart, liver, intestine, kidney and muscle. Rainbow trout and red drum muscle samples were separated into red and white muscle tissue, and rainbow trout were additionally sampled for gas gland and brain tissue ($n = 7$). However, gas gland and brain were not included in the final assessment of CA4a tissue distribution. Owing to the small size of zebrafish, tissues of four adults were combined to generate a single mRNA sample ($n = 4$ samples). GeneJET RNA Purification Kit (Thermo Scientific™) was used to process high-quality zebrafish mRNA. Whole adult zebrafish fish were also processed for mRNA to use as whole-body control ($n = 8$; 4 males, 4 females). Tissue samples for red drum and rainbow trout were processed for total mRNA isolation using TriReagent (Molecular Research Center, Inc.), in accordance with manufacturer protocols. All tissues were homogenized using a rotor-stator homogenizer. All mRNA samples were quantified using an ND-1000 spectrophotometer (Thermo Scientific™) for concentration and purity. Prior to cDNA synthesis, mRNA was subjected to a DNase I treatment following manufacturer guidelines to treat for potential DNA contamination. First strand cDNA was synthesized using 1 μg of total RNA and RevertAid reverse transcriptase (Thermo Scientific™) with no RT controls and stored at −20 °C until use.

Primers published by Lin et al. (2008) were used for zebrafish β-actin and CA4a. Additional primers were designed from published sequences (Table 1): zebrafish CA4b (BC078387.1), zebrafish CA4c (NM_001386230.1), red drum elongation factor 1α (KJ958539.1), red drum CA4 (MT362925.1), rainbow trout CA4a (XM_021624267.2) and rainbow trout CA4b (AY514871.1). We used the same elongation factor 1α primers for red drum and rainbow trout, as they were found to be effective on both species. We attempted to identify additional red drum CA4 isoforms that were not published or available in our transcriptome by designing six primers against the available yellow croaker (*Larimicthys crocea*) sequences (Table 2). Note that yellow croaker and red drum are closely related species of the family Sciaenidae, with most genes exhibiting >90% nucleotide sequence similarity.

We tested for the presence/absence of CA4 isoform mRNA expression using the following RT-PCR protocol: an initial denaturing temperature of 94 °C for 2 min, 40 cycles of 94 °C for 30 s, gradient annealing temperatures (55–63 °C) for 30 s, 72 °C for 1 min, and final extension at 72 °C for 5 min. The resulting PCR products were run on a 2% agarose gel at 70 V and imaged using the BioRad® ChemiDoc. Either β-actin (zebrafish) or ef1α (rainbow trout and red drum) was used for cDNA control, and molecular water was used as negative control (Figs. S1–5).

Table 1
Forward and reverse PCR primers for tissue distribution of membrane-bound carbonic anhydrases.

Species and gene	RT-PCR primers
Red drum ef1a (136 bp)	F 5′ – GTC CGT ACA TGA GGC AGA CTG – 3′
	R 5′ – GTT GCT GGA TGT CCT GCA CG – 3′
Red drum CA4a (103 bp)	F 5′ – CCT TGG GTT TTT CTA TGA GGA GT – 3′
	R 5′ – CAG CGA CGT ATT GTC ATT TGT AG – 3′
Trout ef1a (136 bp)	F 5′ – GTC CGT ACA TGA GGC AGA CTG – 3′
	R 5′ – GTT GCT GGA TGT CCT GCA CG – 3′
Trout CA4a (429 bp)	F 5′ – CAT CGT CCA CAG GAA GAC CCT – 3′
	R 5′– ACC AGA GTT GCA TTG GCA CC – 3′
Trout CA4b (756 bp)	F 5′ – GCA ACT GCA TAG GAC CTG ATG G – 3′
	R 5′ – GAT ACA CCG GGC GAC CAT TCA A – 3′
Zebrafish b-actin (179 bp)	F 5′ – ATT GCT GAC AGG ATG CAG AAG – 3′
	R 5′ – GAT GGT CCA GAC TCA TCG TAC TC – 3′
Zebrafish CA4a (153 bp)	F 5′ – CGG TGT TTG AAA AGC CCA TTC – 3′
	R 5′ – AAT AAA GTC CCA CCG CTG GA – 3′
Zebrafish CA4b (283 bp)	F 5′ – GGG CCA ATT TAG GAT CCA CT – 3′
	R 5′ – TCT GCA TTG CTT TCA GGA TG – 3′
Zebrafish CA4c (220 bp)	F 5′ – TGG CAG TAC TCG CCT TCT TT – 3′
	R 5′ – ACC GTC CAC AAA ACA GCT TC – 3′

Table 2
Yellow croaker degenerate primer pairs used to identify possible red drum CA4 isoforms.

Gene (product size)	RT-PCR primers
Yellow croaker CA4.1 (119 bp)	F 5′ – GSA GAT CAC AGT CTC CAG TYA A – 3′
	R 5′ – SYT GAA GYG CCT TGT ATG TTG – 3′
Yellow croaker CA4.2 (176 bp)	F 5′ – TAT CAG TCC CAG TTC ACC TG – 3′
	R 5′ – CCT CTG AAG ATC TGC TGG TAG – 3′
Yellow croaker CA4.3 (193 bp)	F 5′ – GGT TCT TAC TGT GGA GGA AA – 3′
	R 5′ – TCC ACC TCC ACT YAC TTC AAC – 3′
Yellow croaker CA4.4 (200 bp)	F 5′ – AGC TGT GGA GGA TTA CGC CA – 3′
	R 5′ – GCY CTG TAG TGA CCR GGC AGA – 3′
Yellow croaker CA4.5 (205 bp)	F 5′ – GCA ATG ACA SCA CCT GGC CR – 3′
	R 5′ – TTA CCC CAG TGC AAR TGG AAC – 3′
Yellow croaker CA15.1 (203 bp)	F 5′ – TTA CTT GAA GAG CAC CAY TCT CC – 3′
	R 5′ – GAT GGT GTG GTT ACA CRA TCT G – 3′

Whole adults were used as a positive control for zebrafish. Amplicons for zebrafish CA4a, CA4b, CA4c, rainbow trout CA4a and CA4b, and red drum CA4a were gel extracted (GeneJet PCR Purification Kit; Thermo Scientific) and sequenced to validate gene identity.

3. Results

3.1. Phylogenetic analysis – CA4 isoforms

A phylogenetic tree of 438 fish CA4, CA4-like, CA15, and CA15-like sequences from 77 species in total was constructed using a maximum likelihood-based approach (Fig. 1). The tree was rooted using membrane-bound CA isoforms from four cartilaginous fishes. These were chosen based on their previously described physiological role of chondrichthyan CA4 in CO_2 excretion, which is not observed in teleosts (Gilmour et al., 2007; Gilmour and Perry, 2004, 2010; Gilmour et al., 2002; McMillan et al., 2019). Other chondrichthyan and non-teleost actinopterygiian membrane-bound CAs were distinct from and basal to all teleost CA4s and CA15s, except for reedfish (*Erpetoichthys calabaricus*; non-teleost actinopterygiian species) CA4-like isoforms that were basal to teleost CA15s. All teleost isoforms separated into two distinct sister clades: CA4 and CA15. Both clades include isoforms that are nominally designated as “CA4” and “CA4-like”, but only the CA15 clade includes “CA15” and “CA15-like” sequences. This observation suggests that teleost sequences labeled “CA4” or “CA4-like” within the CA15 clade are, in fact, mislabeled CA15 isoforms.

We defined clades for 4a, 4b, and 4c isoforms according to the original zebrafish nomenclature designated by Lin et al. (2008), and by requiring that each named clade had at least one species of the three teleost subcohorts: Ostariophysi, Protacanthopterygii, and Neoteleostei. CA4a and CA4b clades were more closely related to each other than to CA4c. Interestingly, CA4a of subcohorts Protacanthopterygii and Neoteleostei (both of cohort Euteleostomorpha) grouped more closely with the entire teleost CA4b clade than with CA4a of subcohort Ostariophysi. More simply, Euteleostomorpha CA4a was more closely related to teleost CA4b, both of which were sisters to Ostariophysi CA4a. CA4b and CA4c showed well-defined groupings based on our criterion, with species from each teleost subcohort. Only one species of subcohort Protacanthopterygii (northern pike, *Esox lucius*) was present in the CA4c clade, and this was a predicted sequence assembled from the *E. lucius* genome. Notably, there were no CA4c sequences identified from salmonids.

The coding regions for rainbow trout and red drum CA4a had the highest degree of similarity with each other (68%), followed by their similarity with zebrafish CA4a (60–62%; Table 3). Reflecting results from the phylogenetic analysis, rainbow trout CA4b was most similar to rainbow trout CA4a (61%) and red drum CA4a (59%), followed by zebrafish CA4b (58%) and zebrafish CA4a (57%). Zebrafish CA4c had the least similarity to all other sequences (41–45%). When compared with human CA4, all teleost CA4 sequences had similarities <40%.

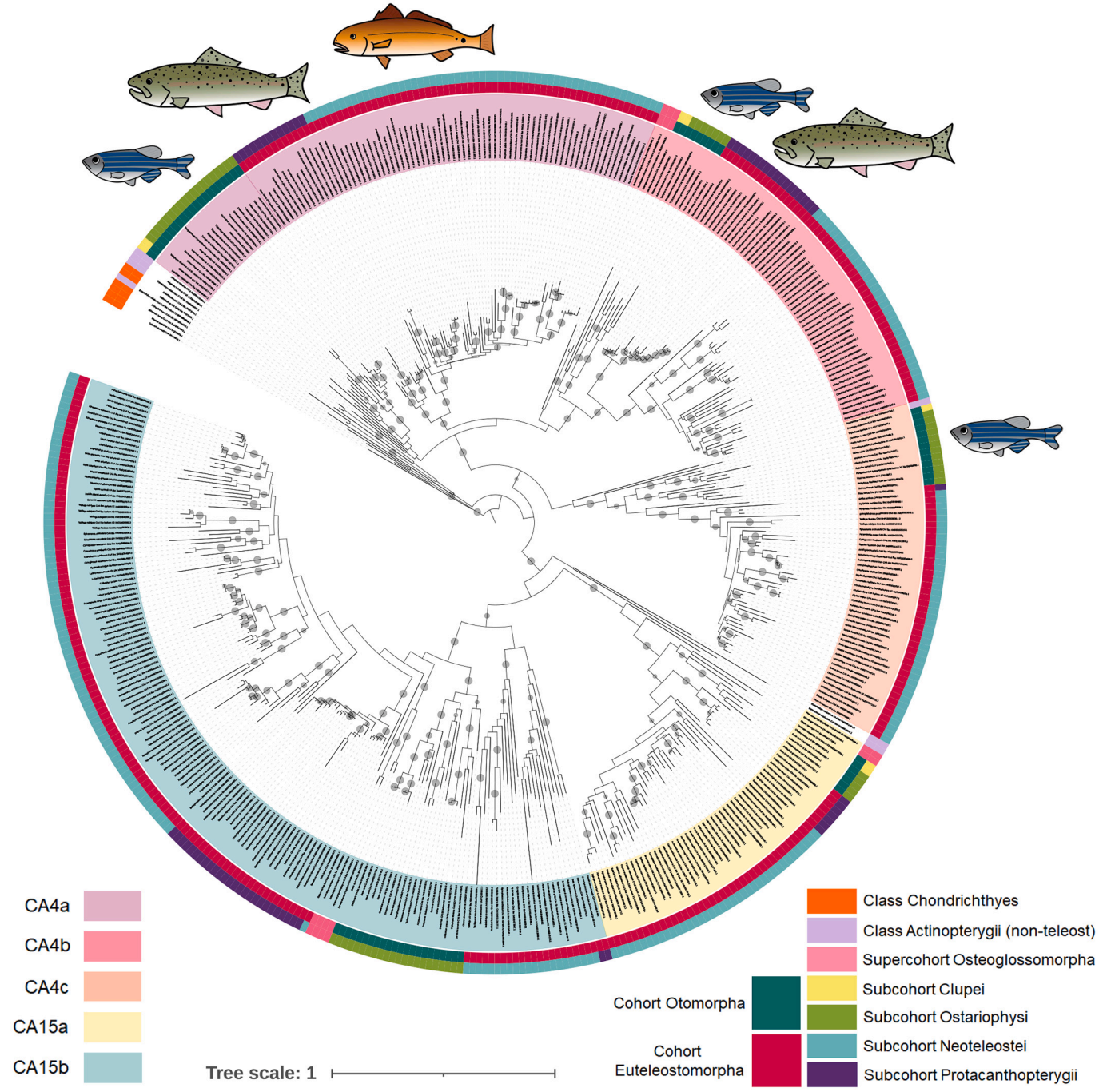

Fig. 1. Phylogeny of the teleost membrane-bound carbonic anhydrase 4 and 15 isoforms from 438 publicly available nucleotide sequences. The tree was generated using IQ-tree v1.6.1 with a best-fit TVM + F + R7 model selected using the Bayesian Information Criterion (BIC). The tree was rooted at the branch of distantly related GPI-linked membrane-bound CAs from class Chondrichthyes. Bootstrap values were calculated using non-parametric bootstrapping with 100 replicates (represented by gray circles, only bootstrap values ≥90 are shown). Circles surrounding the tree denote taxonomy (see legend). Inner circle indicates cohort type: Otomorpha or Euteleostomorpha. Outer circle denotes subcohort type: Ostariophysi, Protacanthopterygii, and Neoteleostei. A smaller number of sequences are presented from subcohort Clupei (cohort Otomorpha), supercohort Osteoglossomorpha, and Class Actinopterygii (non-teleost fish). Sequence names are highlighted at the end of each branch to denote different teleost membrane-bound CA isoform groups (see legend). Fish images demonstrate which species and sequences were targeted for the tissue distribution analysis to predict functional roles. Zebrafish (*Danio rerio*) are representative of subcohort Ostariophysi and were analyzed for CA4a, CA4b, and CA4c tissue distribution. Rainbow trout (*Oncorhynchus mykiss*) are representative of subcohort Protacanthopterygii and were analyzed for CA4a and CA4b tissue distribution. No salmonid CA4c sequences are available, thus no attempts were made to amplify rainbow trout CA4c. Red drum (*Sciaenops ocellatus*) are representative of subcohort Neoteleostei and were analyzed for CA4a only. Attempts to amplify red drum CA4b and CA4c using yellow croaker (*Larimicthys crocea*) sequence information were unsuccessful. The interactive tree can be accessed using: https://itol.embl.de/tree/12862438062151625093609. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Matrix diagram comparing the amino acid sequence identity (%) of zebrafish, rainbow trout, and red drum CA4s, in addition to human CA4. Percent identities were calculated using Clustal Omega Multiple Sequence Alignment (EMBL-EBI; Madeira et al., 2022).

	Zebrafish CA4a	Zebrafish CA4b	Zebrafish CA4c	Rainbow trout CA4a	Rainbow trout CA4b	Red drum CA4a
Human CA4	39.20	37.92	35.67	36.00	37.02	36.42
Zebrafish CA4a		52.00	44.22	60.00	56.70	62.09
Zebrafish CA4b			40.60	50.00	57.79	52.48
Zebrafish CA4c				42.72	44.98	44.08
Rainbow trout CA4a					60.82	68.08
Rainbow trout CA4b						58.90

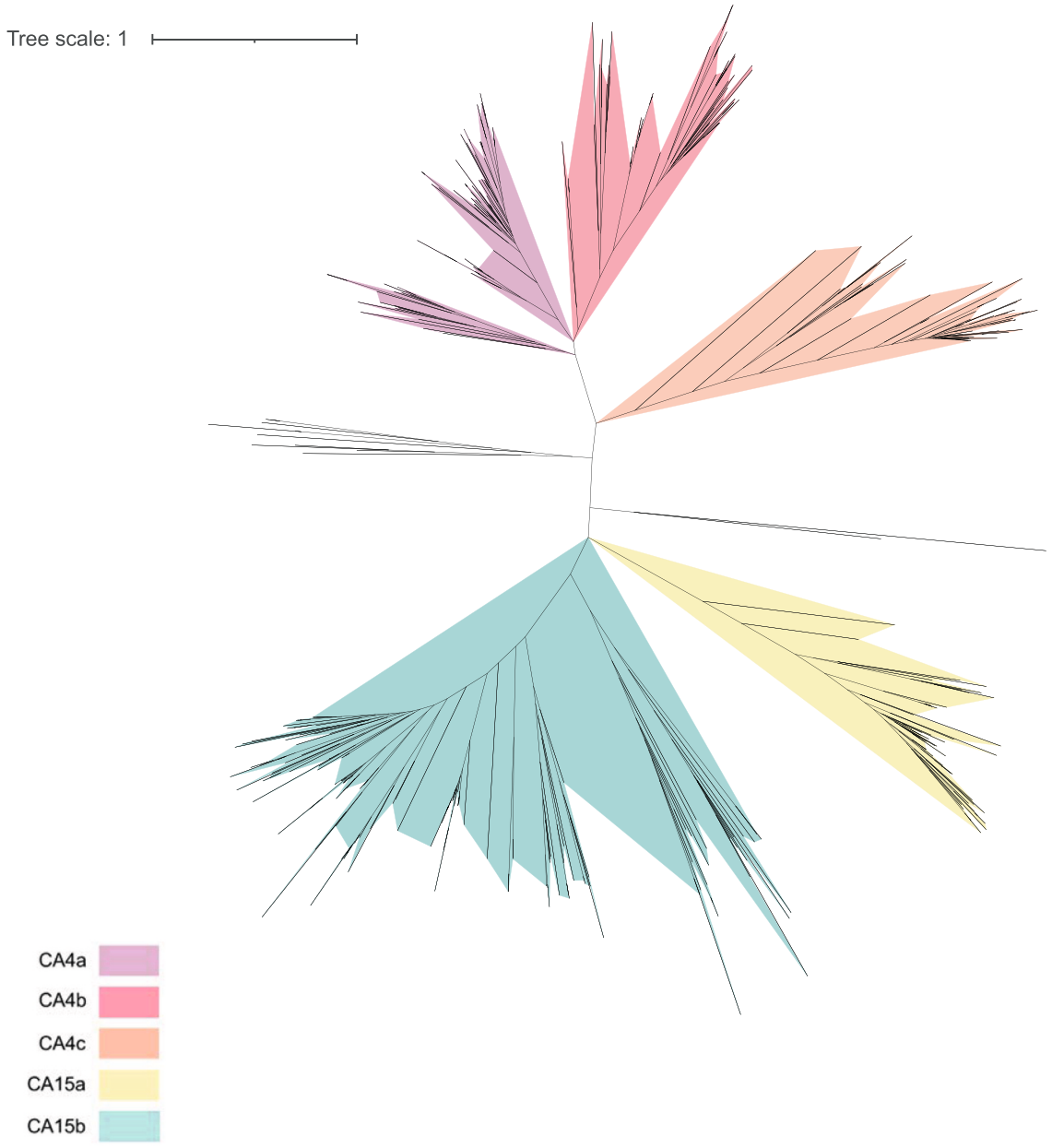

Fig. 2. Unrooted phylogeny of the teleost membrane-bound carbonic anhydrase 4 and 15 isoforms from 438 publicly available nucleotide sequences. The tree was generated using IQ-tree v1.6.1 with a best-fit TVM + F + R7 model selected using the Bayesian Information Criterion (BIC). Bootstrap values were calculated using non-parametric bootstrapping with 100 replicates (represented by gray circles, only bootstrap values ≥90 are shown). Tree branches are highlighted according to the legend to denote different teleost GPI-linked membrane-bound CA isoform groups. Branches that are not highlighted are non-teleost fish CA isoforms. The interactive tree can be accessed using: https://itol.embl.de/tree/12862438062151625093609.

3.2. Phylogenetic analysis – CA15 isoforms

We were primarily interested in the CA4 isoforms, which we targeted for tissue distribution experiments (see below). Nevertheless, our analysis also provides important information on CA4-like, CA15, and CA15-like isoforms. There appear to be at least two distinct groups of CA15 based on our criterion that each isoform clade must have species from three major teleost lineages. However, the tight relationship of the previously identified zebrafish CA15s (a, b, c) *within* one of these two large CA15 clades negates our ability to label these groups according to the nomenclature proposed by Lin et al. (2008). For example, zebrafish CA15a and CA15c genes originally published by Lin et al. (2008) exhibit 80% amino acid sequence similarity (using the National Center for Biotechnology Information Basic Local Alignment Search Tool BLASTP 2.13.0+ against database nr). Consequently, we nominally designated the two large groups of CA15 as CA15a and CA15b based on their phylogenetic relationships, where CA15a includes previously identified CA15a, b, and c. An unrooted tree was used to visualize distinctions among isoform clades (Fig. 2) and suggests there may be two groups *within* what we nominally designated as CA15b. However, this potential third group consists solely of teleosts from subcohorts Protacanthopterygii and Neoteleostei, including two salmonids. Without representative species from the subcohort Ostariophysi, we did not separately classify this potential third group and suggest that until functional work is conducted across CA15 isoforms, all CA15 isoforms should be presently classified as either CA15a or CA15b.

3.3. CA4 tissue distribution

To provide some insight into the functional significance of the three distinct CA4 isoforms (originally described by Lin et al. (2008) and confirmed in this study), we assessed their relative distribution across tissues using mRNA expression. Predicted functions were assigned to each tissue based on the available literature (Fig. 3). Three species of teleosts (zebrafish, *Danio rerio*; rainbow trout, *Oncorhynchus mykiss*; and red drum, *Sciaenops ocellatus*) were used to represent major teleost lineages (Ostariophysi, Protacanthopterygii, and Neoteleostei, respectively).

Surprisingly, we found that the relative distribution of CA4a varied across the representative species (Figs. 4 and 5). In zebrafish, CA4a expression was isolated to the combined red and white muscle sample with no expression evident in other tissues. CA4a was also found in both red and white muscle in rainbow trout, with additional expression detected in the heart and liver. Red drum similarly expressed CA4a in the muscle, heart, and liver, but also demonstrated strong expression in the kidney and inconsistent expression in the gill and intestine (see Fig. 4 for details). Notably, there was no CA4a expression in the kidney of either zebrafish or rainbow trout in any sample tested.

The distribution of CA4b also varied among the three species (Fig. 4). CA4b expression was not detected in any specific tissue in zebrafish, and in fact this isoform was only sporadically identified in whole animal homogenate mRNA samples (2 of 8 samples, Fig. S1). Of the three, the rainbow trout gave the clearest pattern with CA4b being primarily expressed in the kidney. Similar to zebrafish, CA4b was not isolated from red drum using the tissue panel in question. Note that red drum CA4b primers (Table 2) were designed against the known CA4b sequence for yellow croaker (*Larimichthys crocea*), a species that is very closely related to red drum, and which shows very high sequence homology with red drum in other CA isoforms.

Phylogenetic analysis (Figs. 1 and 2) suggested that CA4c could be found only in zebrafish and red drum, and CA4c sequences were almost ubiquitously absent from Protacanthopterygii species. Therefore, no attempts to clone a CA4c gene from rainbow trout were undertaken. For zebrafish, CA4c was not identified in any specific tissue, nor was it expressed to a detectable level in the whole animal homogenate samples. Although a CA4c sequence was identified for yellow croaker, attempts to amplify the red drum sequence using yellow croaker primers (Table 2) were unsuccessful.

4. Discussion

Plasma-accessible membrane-bound CAs – specifically CA4 type isoforms – have been of particular interest in recent years due to their emerging roles in tissue O_2 delivery in fishes (Harter and Brauner, 2017). However, with the number of described GPI-linked isoforms in teleosts, and the imperfect system of CA isoform nomenclature, it has been challenging to properly ascribe physiological function to particular CA isoforms across fish taxa. Here we present a phylogenetic analysis of GPI-linked CAs coupled with mRNA expression patterns across tissues for representative species to provide an organizational framework for future CA4 and CA4-like isoform research in fishes.

Lin et al. (2008) first established the idea that fishes may exhibit functional specialization of CA4 isoforms by describing the presence of multiple GPI-anchored membrane-bound CA isoforms in the zebrafish genome. Yet since their initial work, there has been little effort to pursue this idea or to ask whether it applies beyond zebrafish. Using the plethora of sequence information currently available, we performed a comprehensive phylogenetic analysis to explore the suite of CA4

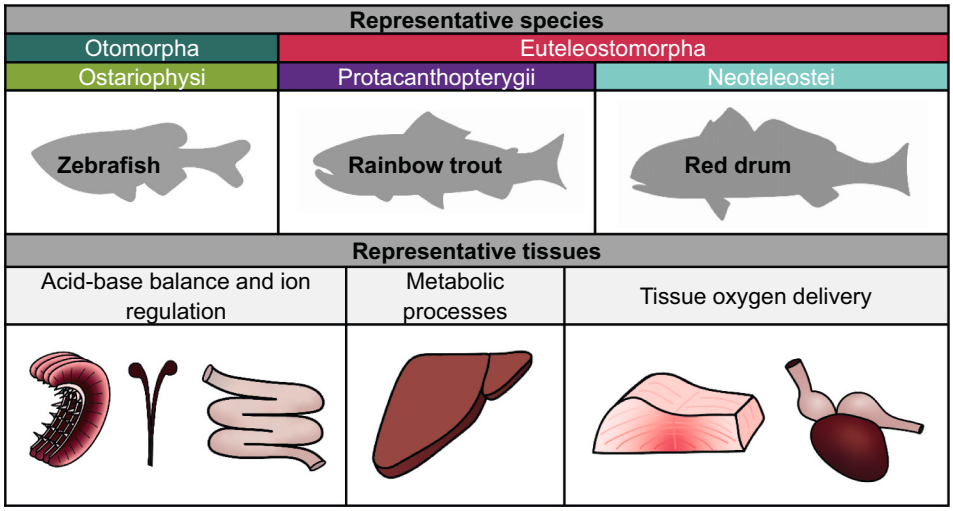

Fig. 3. Representative species and tissues used for tissue distribution and predicted function analyses. Species from two cohorts (Otomorpha and Euteleostomorpha) and three subcohorts (Ostartiophysi, Neoteleostei, Protacanthopterygii) were used to represent three distinct teleost lineages. Zebrafish (*Danio rerio*) represented subcohort Ostartiophysi from cohort Otomorpha. Rainbow trout (*Oncorhynchus mykiss*) represented subcohort Protacanthopterygii and red drum (*Sciaenops ocellatus*) represented subcohort Neoteleostei, both from cohort Euteleostomorpha. Although tissue distribution does not predict function, previous literature allowed us to group representative tissues into predicted functional groups: acid-base balance and ion regulation (gills, kidney, intestine), metabolic processes (liver), and tissue oxygen delivery (muscle, heart). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

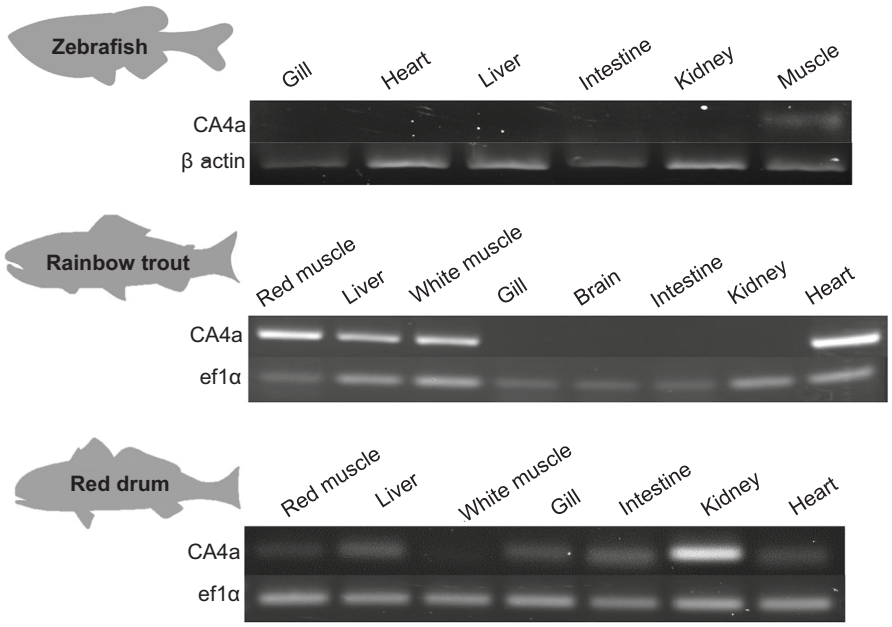

Fig. 4. Representative gel electrophoresis images of CA4a tissue distribution. For zebrafish (*Danio rerio*), CA4a was only present in the muscle (4 out of 4 samples). For rainbow trout (*Oncorhynchus mykiss*), CA4a was present in the red muscle (7 out of 7 samples), liver (7 out of 7 samples), white muscle (5 out of 7 samples), and heart (7 out of 7 samples). For red drum (*Sciaenops ocellatus*), CA4a was present in the red muscle (4 out of 4 samples), liver (4 out of 4 samples), white muscle (3 out of 4 samples), gill (4 out of 4 samples), intestine (2 out of 4 samples), kidney (4 out of 4 samples), and heart (4 out of 4 samples). See Supplemental Material for all gel electrophoresis images. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

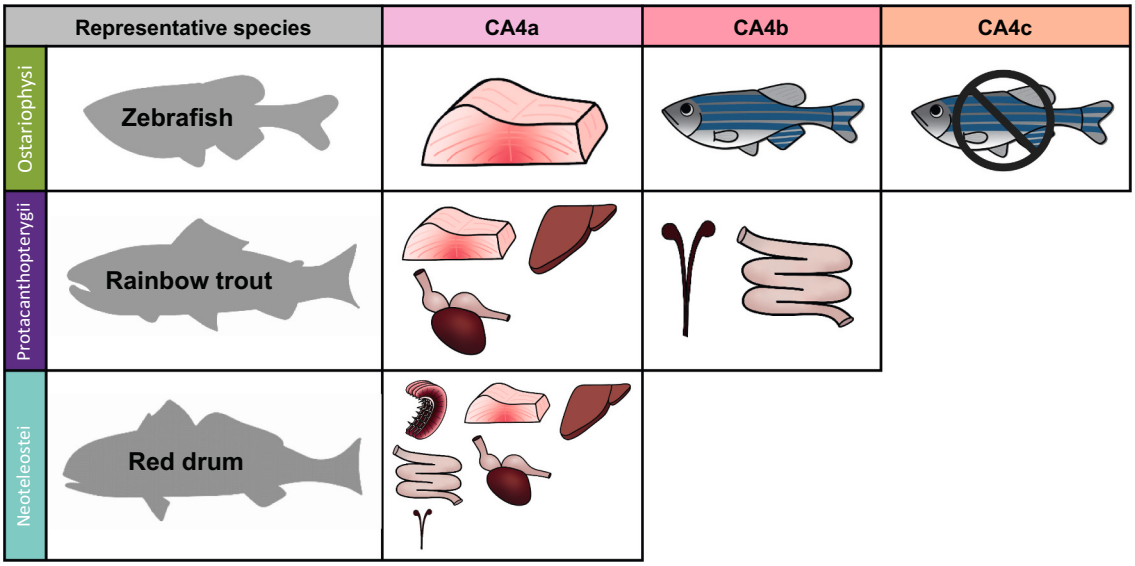

Fig. 5. Overview of CA4 tissue distribution for species representative of three major teleost lineages. For zebrafish (*Danio rerio*) CA4a was only present in the muscle, CA4b was only present in whole-body samples, and CA4c was not detected. For rainbow trout (*Oncorhynchus mykiss*), CA4a was present in the muscle, heart, and liver, and CA4b was present only in the kidney and intestine. No salmonid CA4c sequences are available, thus no attempts were made to amplify rainbow trout CA4c. For red drum (*Sciaenops ocellatus*), CA4a was present in the gill, kidney, intestine, muscle, heart, and liver. Attempts to amplify red drum CA4b and CA4c using yellow croaker (*Larimichthys crocea*) sequence information were unsuccessful. Zebrafish and rainbow trout demonstrated functional divergence of CA4a and CA4b isoforms (i.e., isoforms were detected in different tissues without overlap). CA4a may play a role in tissue oxygen delivery in all species tested (presence in muscle and heart), whereas CA4b shows a potential role in acid-base and ion regulation in rainbow trout (presence in kidney and intestine). Conversely, red drum expressed CA4a in all tissues tested and did not demonstrate functional divergence. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

isoforms expressed in teleosts. We demonstrated that teleost CA4 sequences could be divided into three well-supported isoforms (CA4a, CA4b, and CA4c) confirming the original designation proposed by Lin et al. (2008). Of these, CA4a and CA4b are most closely related, whereas CA4c is basal to both of these isoforms. It should be noted that duplications of sequences are present *within* groups of isoforms – for example, three CA4a sequences exist for rainbow trout (*Oncorhynchus mykiss*) *within* the Euteleosteomorpha CA4a group. However, these sequences were derived from genome assembly and although they are not identical, the sequences have high percent identities and may not represent three separate CA4a isoforms. Therefore, for the purposes of our analysis, duplications *within* isoform groups were not considered to be reflective of multiple isoforms, but rather of differences in methodology. For example, in some instances separate research groups have published similar sequences from the same species, which can contribute to apparent duplications *within* isoform groups. Given these assumptions, our data indicate that teleost CA4 can be categorized into three isoforms: CA4a, CA4b, and CA4c. However, not every species

possesses all three isoforms. The only CA4c sequence from subcohort Protacanthopterygii was a predicted sequence from *Esox lucius* (northern pike) despite the fact that several salmonid genomes have been sequenced. Thus, CA4c may be completely absent from salmonids.

The main motivation behind this was work was to establish candidate CA4 isoforms that may contribute to tissue O_2 extraction in teleost fishes. Briefly, recent work demonstrated that membrane-bound CA4 in aerobically-demanding tissues can act to short circuit RBC pH regulation to the benefit of tissue O_2 delivery. Typically, RBC proteins (specifically, β-NHE) regulate pH under stressful conditions that elicit catecholamine mobilization, assuring that pH-sensitive hemoglobins can maintain O_2 binding at the gill. The presence of CA4 at the tissue causes a localized extracellular acidosis that increases local O_2 partial pressure gradients, which overwhelms RBC pH regulation, reduces the affinity of hemoglobin for O_2, and improves O_2 offloading from hemoglobin (Brauner and Harter, 2017; Harter and Brauner, 2017, 2020). To date, this mechanism has been demonstrated to improve tissue O_2 extraction at the eye (Damsgaard et al., 2020), red muscle (Harter et al., 2019; Rummer and Brauner, 2011; Rummer and Brauner, 2015; Rummer et al., 2013), and heart (Alderman et al., 2016) of salmonid fishes, but has not yet been extended to other fish families. Our data suggest that the isoform responsible for this mechanism is CA4a, which was expressed in the muscle of all three species tested, and the heart of both Euteleosteomorpha species (rainbow trout and red drum). Although functional studies on the involvement of plasma-accessible membrane-bound CA in O_2 delivery have so far been confined to salmonids, our work may suggest that this mechanism is more broadly distributed. For example, zebrafish have both a moderate Root effect and β-NHE activity (Berenbrink et al., 2005). When coupled with CA4a localization in the muscle, heart, and eye (Lin et al., 2008), there is clear potential for zebrafish to benefit from enhanced tissue O_2 extraction. This is also the case for red drum, which previously were shown to possess a large Root effect along with β-NHE activity, as well as RBC CA activity that significantly contributes to Hb-O_2 offloading (Dichiera and Esbaugh, 2020).

We are not suggesting that CA4a expression in a tissue is, on its own, sufficient evidence for a role for CA4a in O_2 extraction. Instead, our results suggest that the biochemical *potential* for enhanced tissue O_2 extraction is widespread in teleosts, and we propose CA4a as a likely target protein for functional studies related to tissue O_2 delivery. While enhanced O_2 delivery may be a common feature for many salmonids even at steady state conditions (Shu et al., 2018), additional roles of CA4 in muscle have been previously described. CA4 oriented extracellularly on capillary walls may facilitate CO_2 transport and maintain pH equilibration, similar to CA4 in mammalian pulmonary capillary beds (reviewed by Geers and Gros, 2000). Additionally, CA4 has been found on the extracellular surface of the sarcolemma and sarcoplasmic reticulum, facilitating CO_2 excretion and ammonia efflux during steady state conditions and lactate efflux during exercise (Henry et al., 1997b; Wang et al., 1998). Thus, biochemical characterization of CA4a in the muscle of zebrafish and red drum is needed to fully understand the physiological role this isoform plays, whether in enhanced O_2 extraction, CO_2 excretion, ammonia and lactate efflux, or all of the above.

Interestingly, all red drum gill samples expressed CA4a, which is not consistent with the hypothesis of tissue O_2 extraction, where plasma-accessible CA isoforms in the gill could compromise gill O_2 uptake during acidification. Teleost branchial CA is thought to be exclusively cytoplasmic, often in conjunction with powerful plasma CA inhibitors (Henry et al., 1997a). Conversely, functional membrane-bound plasma-accessible CA4 found in elasmobranch gills is used to excrete CO_2, but these fish generally do not have plasma CA inhibitors, pH-sensitive Hb or β-NHE activity necessary for tissue O_2 extraction (Berenbrink et al., 2005; Gilmour et al., 2007; Gilmour and Perry, 2004, 2010; Gilmour et al., 2002; McMillan et al., 2019). It is possible that CA4a expressed in red drum is not plasma-accessible at the gills but instead oriented towards the external environment (e.g., CA15a in zebrafish gills) where it would assist acid-trapping mechanisms to promote ammonia excretion (Wright and Wood, 2009), facilitate CO_2 excretion, or otherwise contribute to apical ion exchange processes (Hwang, 2009; Hwang and Perry, 2010; Hwang et al., 2011). Further biochemical studies are needed to verify the role branchial CA4a plays in red drum, including the characterization of red drum plasma CA inhibitors and their effects on plasma-accessible CA4.

The relatively strong expression of CA4a in the liver of rainbow trout and red drum was somewhat surprising. The available data from mammals suggest that CA9 is the sole membrane-associated CA isoform in this tissue (Purkerson and Schwartz, 2005). This isoform is expressed on both the basolateral and apical membranes of hepatocytes, with the active site in the extracellular environment, where it is believed to contribute to pH balance and ion transport among the hepatocytes, sinusoids, and bile capillaries (Parkkila et al., 2002). The discovery of CA4a in rainbow trout and red drum liver could suggest that CA4a has adopted this function in fishes. Alternatively, the liver – as a metabolically active tissue – may also benefit from elevated O_2 extraction efficiency. For example, in mammals the resting metabolic rate of the liver is comparable to that of the brain (Wang et al., 2010). It is also interesting that CA4a expression in the liver differed across species, as both rainbow trout and red drum had relatively robust expression whereas zebrafish did not (Lin et al., 2008).

Membrane-bound CA has also been implicated in ion and acid-base regulation in the kidneys and intestine. In the kidney of freshwater fish, the enzyme is oriented into the nephron lumen where it is accessible to the glomerular filtrate and works in tandem with cytoplasmic CA to regulate HCO_3^- reabsorption to maintain acid-base status (Gilmour and Perry, 2009). In the intestine of seawater-acclimated fish, luminal CA4 is similarly thought to help maintain HCO_3^- gradients for anion exchange, and further promote the absorption of osmolytes through the precipitation of $CaCO_3$ (Grosell et al., 2009a). We found that rainbow trout did not express CA4a in the kidney and intestine and instead exclusively expressed CA4b. Thus, we suggest previous research that has explored the role of CA4 in rainbow trout renal HCO_3^- reabsorption (Georgalis et al., 2006) and intestinal acid excretion (Georgalis et al., 2006; Grosell et al., 2009a; Grosell et al., 2007) can be attributed to the CA4b isoform. It is interesting to note that neither our study, nor Lin et al. (2008) found CA4 or CA4-like isoforms in the freshwater zebrafish kidney. In zebrafish, CA4b was present in the adult whole-body homogenate, indicating expression in tissues not specifically examined (e.g., swimbladder or brain). Importantly, zebrafish CA4b was not expressed in any of the tissues that expressed CA4a, suggesting that both zebrafish and rainbow trout CA4b have separate functional roles from that of CA4a.

Interestingly, we only found one CA4 isoform in red drum: CA4a. However, the tissue distribution analysis suggests that red drum CA4a is a "jack-of-all-trades" protein, with potential to contribute to acid-base balance and ion regulation, metabolic processes, and tissue O_2 delivery. In addition to muscle, all red drum tested expressed some amount of CA4a in the kidney. It is important to note that in marine fishes, such as red drum, the kidney is of minor importance for filtration, as compared to primarily freshwater fishes such as rainbow trout and zebrafish. However, red drum are euryhaline fish that can be quickly transferred to freshwater with minimal physiological impact (Watson et al., 2014). It remains unknown whether red drum increase expression of CA4b in response to freshwater acclimation and future studies exploring the functional significance of CA4a versus CA4b for renal function in fishes would be beneficial.

Despite considerable effort dedicated to tissue distribution analyses, CA4c expression was not detected in any tissue of any of the three species, nor in the adult zebrafish whole body homogenate samples. The CA4c expression profile likely is dependent on the selected tissue panel, the life stage of the organism, and the environment to which the fish was acclimated. As such, we are not suggesting that CA4c lacks physiological significance in fishes, but rather that its function lies beyond those

studied here. Prior work on CA isoforms in lamprey demonstrated a shift in predominant cytoplasmic isoform identity across development – the dominant isoform changed from CA16 during the early ammocoete phase to CA18 during the post-metamorphic stage (Ferreira-Martins et al., 2016). It is possible that something similar may occur for CA4c whereby its role is limited to a particular stage of development, or that it may play a unique role in a tissue not studied here.

As mentioned previously, our focus was primarily on CA4 and CA4-like isoforms owing to their high activity kinetics and putative physiological roles in fishes; however, our work also provides important insight into the CA15 clade, which included many "CA4" or "CA4-like" sequences we now suspect have been incorrectly annotated. There appear to be at least two CA15 isoforms, but this conclusion should be treated carefully as the phylogenetic pattern across teleosts is poorly resolved. While CA15 isoforms are reported to have low activity in mammals (Hilvo et al., 2005), there have been no attempts to biochemically characterize CA15 from fishes. In zebrafish, CA15a was found primarily in the gill of adult zebrafish and was demonstrated to function in epithelial acid excretion and Na^+ uptake in the cutaneous ionocytes of larval fish (analogous to adult gill). However, this original work from Lin et al. (2008) is one of just a few studies on fish CA15 to our knowledge, and all have been conducted on zebrafish (e.g., Ito et al., 2013; Pan et al., 2022). CA15 isoforms therefore present an exciting avenue of research to further improve our understanding of membrane-bound CA contributions to respiratory gas exchange, acid-base balance, and ion regulation.

In conclusion, our work reinforces the premise that membrane-bound CA4 isoforms in fishes can be divided into three distinct isoforms, with CA4a likely being responsible for the emerging role of plasma-accessible membrane-bound CA in facilitating O_2 extraction. More importantly, our work suggests that the functional significance of the respective isoforms varies widely across fish taxa. This was particularly noteworthy in kidney, where the available information suggests that CA4b in Protacanthopterygii contributes to renal pH balance and ion regulation, whereas CA4a takes this role in Neoteleostei. Surprisingly, no CA4 isoform was found in the kidney of zebrafish, suggesting that this role may have been taken by CA15. Although we acknowledge that our mRNA expression observations are based on a single representative species for each subcohort, and as such should be interpreted with some caution, our findings nonetheless provide a useful framework to guide future research and help elucidate the physiological significance of CA4 isoforms in fishes.

Contributions

A.J.E. conceptualized and supervised the project. A.M.D. conducted the investigation including tissue distribution, data mining, and initial phylogenetic analyses. K.M.G. provided samples for tissue distribution analyses. V.D.A. conducted the phylogenetic analyses, and B.J.B. provided resources for phylogenetic analyses. A.M.D., V.D.A., and A.J.E. analyzed the results. A.M.D. and V.D.A. created the data visualization and A.M.D. wrote the initial manuscript draft. All authors reviewed the manuscript.

Funding information

This research was made possible by a grant from the National Science Foundation awarded to A.J.E. (#2002549), and A.M.D. was supported by the University of Texas at Austin Stengl-Wyer Graduate Fellowship and National Science Foundation Postdoctoral Research Fellowship in Biology (#2109765).

Declaration of Competing Interest

The authors declare no competing interests.

Data availability

NCBI accession numbers for all sequences used in the phylogenetic analysis have been deposited in Figshare (https://doi.org/10.6084/m9.figshare.20751904.v1).

Acknowledgments

The authors would like to thank previous undergraduates Y. K. Pan and A. Johnson for their preliminary work on red drum and rainbow trout CA4 distribution, and S. J. Brandl for exploratory phylogenetic and ecological analyses.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpa.2023.111368.

References

Alderman, S.L., Harter, T.S., Wilson, J.M., Supuran, C.T., Farrell, A.P., Brauner, C.J., 2016. Evidence for a plasma-accessible carbonic anhydrase in the lumen of salmon heart that may enhance oxygen delivery to the myocardium. J. Exp. Biol. 219, 719–724.

Banerjee, S., Deshpande, P.A., 2016. On origin and evolution of carbonic anhydrase isozymes: a phylogenetic analysis from whole-enzyme to active site. Comput. Biol. Chem. 61, 121–129.

Berenbrink, M., Koldkjær, P., Kepp, O., Cossins, A.R., 2005. Evolution of oxygen secretion in fishes and the emergence of a complex physiological system. Science 307, 1752–1757.

Berthelot, C., Brunet, F., Chalopin, D., Juanchich, A., Bernard, M., Noël, B., Bento, P., Da Silva, C., Labadie, K., Alberti, A., 2014. The rainbow trout genome provides novel insights into evolution after whole-genome duplication in vertebrates. Nat. Commun. 5, 1–10.

Betancur-R, R., Wiley, E.O., Arratia, G., Acero, A., Bailly, N., Miya, M., Lecointre, G., Ortí, G., 2017. Phylogenetic classification of bony fishes. BMC Evol. Biol. 17, 162.

Brauner, C.J., Harter, T.S., 2017. Beyond just hemoglobin: red blood cell potentiation of hemoglobin-oxygen unloading in fish. J. Appl. Physiol. 123, 935–941.

Damsgaard, C., Lauridsen, H., Harter, T.S., Kwan, G.T., Thomsen, J.S., Funder, A.M., Supuran, C.T., Tresguerres, M., Matthews, P.G., Brauner, C.J., 2020. A novel acidification mechanism for greatly enhanced oxygen supply to the fish retina. Elife 9, e58995.

Dichiera, A.M., Esbaugh, A.J., 2020. Red blood cell carbonic anhydrase mediates oxygen delivery via the root effect in red drum. J. Exp. Biol. 223.

Esbaugh, A., Tufts, B., 2006. The structure and function of carbonic anhydrase isozymes in the respiratory system of vertebrates. Respir. Physiol. Neurobiol. 154, 185–198.

Esbaugh, A., Lund, S., Tufts, B., 2004. Comparative physiology and molecular analysis of carbonic anhydrase from the red blood cells of teleost fish. J. Comp. Physiol. B. 174, 429–438.

Esbaugh, A., Perry, S., Bayaa, M., Georgalis, T., Nickerson, J., Tufts, B., Gilmour, K., 2005. Cytoplasmic carbonic anhydrase isozymes in rainbow trout *Oncorhynchus mykiss*: comparative physiology and molecular evolution. J. Exp. Biol. 208, 1951–1961.

Ferreira-Martins, D., McCormick, S., Campos, A., Lopes-Marques, M., Osório, H., Coimbra, J., Castro, L., Wilson, J.M., 2016. A cytosolic carbonic anhydrase molecular switch occurs in the gills of metamorphic sea lamprey. Sci. Rep. 6, 33954.

Geers, C., Gros, G., 2000. Carbon dioxide transport and carbonic anhydrase in blood and muscle. Physiol. Rev. 80, 681–715.

Georgalis, T., Gilmour, K., Yorston, J., Perry, S., 2006. Roles of cytosolic and membrane-bound carbonic anhydrase in renal control of acid-base balance in 15 rainbow trout, *Oncorhynchus mykiss*. Am. J. Physiol. Ren. Physiol. 291, F407–F421.

Gervais, M.R., Tufts, B.L., 1998. Evidence for membrane-bound carbonic anhydrase in the air bladder of bowfin (*Amia calva*), a primitive air-breathing fish. J. Exp. Biol. 201, 2205–2212.

Gilmour, K., 2010. Perspectives on carbonic anhydrase. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 157, 193–197.

Gilmour, K.M., Perry, S.F., 2004. Branchial membrane-associated carbonic anhydrase activity maintains CO_2 excretion in severely anemic dogfish. Am. J. Phys. Regul. Integr. Comp. Phys. 286, R1138–R1148.

Gilmour, K.M., Perry, S.F., 2009. Carbonic anhydrase and acid–base regulation in fish. J. Exp. Biol. 212, 1647–1661.

Gilmour, K.M., Perry, S.F., 2010. Gas transfer in dogfish: a unique model of CO_2 excretion. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 155, 476–485.

Gilmour, K.M., Shah, B., Szebedinszky, C., 2002. An investigation of carbonic anhydrase activity in the gills and blood plasma of brown bullhead (*Ameiurus nebulosus*), longnose skate (*Raja rhina*), and spotted raffish (*Hydrolagus colliei*). J. Comp. Physiol. B. 172, 77–86.

Gilmour, K., Bayaa, M., Kenney, L., McNeill, B., Perry, S., 2007. Type IV carbonic anhydrase is present in the gills of spiny dogfish (*Squalus acanthias*). Am. J. Physiol. Reg. Integr. Comp. Physiol. 292, R556–R567.

Grosell, M., Gilmour, K.M., Perry, S.F., 2007. Intestinal carbonic anhydrase, bicarbonate, and proton carriers play a role in the acclimation of rainbow trout to seawater. Am. J. Physiol. Reg. Integr. Comp. Physiol. 293, R2099–R2111.
Grosell, M., Genz, J., Taylor, J., Perry, S., Gilmour, K., 2009a. The involvement of H^+-ATPase and carbonic anhydrase in intestinal HCO_3–secretion in seawater-acclimated rainbow trout. J. Exp. Biol. 212, 1940–1948.
Grosell, M., Mager, E., Williams, C., Taylor, J., 2009b. High rates of HCO_3–secretion and cl–absorption against adverse gradients in the marine teleost intestine: the involvement of an electrogenic anion exchanger and H^+-pump metabolon? J. Exp. Biol. 212, 1684–1696.
Harter, T.S., Brauner, C.J., 2017. The O_2 and CO_2 transport system in Teleosts and the specialized mechanisms that enhance Hb–O_2 unloading to tissues. In: Fish Physiology, 36, pp. 1–106.
Harter, T.S., Brauner, C.J., 2020. Teleost red blood cells actively enhance the passive diffusion of oxygen that was discovered by August Krogh. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 110855.
Harter, T., Zanuzzo, F., Supuran, C., Gamperl, A., Brauner, C., 2019. Functional support for a novel mechanism that enhances tissue oxygen extraction in a teleost fish. Proc. R. Soc. B 286, 20190339.
Henry, R.P., Swenson, E.R., 2000. The distribution and physiological significance of carbonic anhydrase in vertebrate gas exchange organs. Respir. Physiol. 121, 1–12.
Henry, R.P., Gilmour, K.M., Wood, C.M., Perry, S.F., 1997a. Extracellular carbonic anhydrase activity and carbonic anhydrase inhibitors in the circulatory system of fish. Physiol. Zool. 70, 650–659.
Henry, R.P., Wang, Y., Wood, C.M., 1997b. Carbonic anhydrase facilitates CO_2 and NH_3 transport across the sarcolemma of trout white muscle. Am. J. Physiol. Reg. Integr. Comp. Physiol. 272, R1754–R1761.
Hilvo, M., Tolvanen, M., Clark, A., Shen, B., Shah, G.N., Waheed, A., Halmi, P., Hänninen, M., Hämäläinen, J.M., Vihinen, M., 2005. Characterization of CA XV, a new GPI-anchored form of carbonic anhydrase. Biochem. J. 392, 83–92.
Hughes, L.C., Ortí, G., Huang, Y., Sun, Y., Baldwin, C.C., Thompson, A.W., Arcila, D., Betancur-R, R., Li, C., Becker, L., 2018. Comprehensive phylogeny of ray-finned fishes (Actinopterygii) based on transcriptomic and genomic data. Proc. Natl. Acad. Sci. 115, 6249–6254.
Hwang, P.P., 2009. Ion uptake and acid secretion in zebrafish (*Danio rerio*). J. Exp. Biol. 212, 1745–1752.
Hwang, P.P., Perry, S.F., 2010. Ionic and acid–base regulation. In: Fish Physiology, 29, pp. 311–344.
Hwang, P.P., Lee, T.H., Lin, L.Y., 2011. Ion regulation in fish gills: recent progress in the cellular and molecular mechanisms. Am. J. Physiol. Reg. Integr. Comp. Physiol. 301, R28–R47.
Ito, Y., Kobayashi, S., Nakamura, N., Miyagi, H., Esaki, M., Hoshijima, K., Hirose, S., 2013. Close Association of Carbonic Anhydrase (CA2a and CA15a), Na(+)/H(+) exchanger (Nhe3b), and Ammonia transporter Rhcg1 in zebrafish Ionocytes responsible for Na(+) uptake. Front. Physiol. 4, 59.
Katoh, K., Standley, D.M., 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol. Biol. Evol. 30, 772–780.
Letunic, I., Bork, P., 2019. Interactive tree of life (iTOL) v4: recent updates and new developments. Nucleic Acids Res. 47, W256–W259.
Lin, T.-Y., Liao, B.-K., Horng, J.-L., Yan, J.-J., Hsiao, C.-D., Hwang, P.-P., 2008. Carbonic anhydrase 2-like a and 15a are involved in acid-base regulation and Na^+ uptake in zebrafish H^+-ATPase-rich cells. Am. J. Phys. Cell Phys. 294, C1250–C1260.
Madeira, F., Pearce, M., Tivey, A.R., Basutkar, P., Lee, J., Edbali, O., Madhusoodanan, N., Kolesnikov, A., Lopez, R., 2022. Search and sequence analysis tools services from EMBL-EBI in 2022. Nucleic Acids Res. 50, W276–W279.
McMillan, O.J., Dichiera, A.M., Harter, T.S., Wilson, J.M., Esbaugh, A.J., Brauner, C.J., 2019. Blood and gill carbonic anhydrase in the context of a chondrichthyan model of CO_2 excretion. Physiol. Biochem. Zool. 92, 554–566.
Pan, W., Godoy, R.S., Cook, D.P., Scott, A.L., Nurse, C.A., Jonz, M.G., 2022. Single-cell transcriptomic analysis of neuroepithelial cells and other cell types of the gills of zebrafish (*Danio rerio*) exposed to hypoxia. Sci. Rep. 12, 10144.
Parkkila, S., Kivelä, A.J., Kaunisto, K., Parkkila, A.-K., Hakkola, J., Rajaniemi, H., Waheed, A., Sly, W.S., 2002. The plasma membrane carbonic anhydrase in murine hepatocytes identified as isozyme XIV. BMC Gastroenterol. 2, 1–7.
Pelis, R.M., Goldmeyer, J.E., Crivello, J., Renfro, J.L., 2003. Cortisol alters carbonic anhydrase-mediated renal sulfate secretion. Am. J. Physiol. Reg. Integr. Comp. Physiol. 285, R1430–R1438.
Pelster, B., 1995. Mechanisms of acid release in isolated gas gland cells of the European eel *Anguilla anguilla*. Am. J. Physiol. Reg. Integr. Comp. Physiol. 269, R793–R799.
Purkerson, J.M., Schwartz, G.J., 2005. Expression of membrane-associated carbonic anhydrase isoforms IV, IX, XII, and XIV in the rabbit: induction of CA IV and IX during maturation. Am. J. Physiol. Reg. Integr. Comp. Physiol. 288, R1256–R1263.
Randall, D., Rummer, J., Wilson, J., Wang, S., Brauner, C., 2014. A unique mode of tissue oxygenation and the adaptive radiation of teleost fishes. J. Exp. Biol. 217, 1205–1214.
Rummer, J.L., Brauner, C.J., 2011. Plasma-accessible carbonic anhydrase at the tissue of a teleost fish may greatly enhance oxygen delivery: *in vitro* evidence in rainbow trout, *Oncorhynchus mykiss*. J. Exp. Biol. 214, 2319–2328.
Rummer, J.L., Brauner, C.J., 2015. Root effect haemoglobins in fish may greatly enhance general oxygen delivery relative to other vertebrates. PLoS One 10, e0139477.
Rummer, J.L., McKenzie, D.J., Innocenti, A., Supuran, C.T., Brauner, C.J., 2013. Root effect hemoglobin may have evolved to enhance general tissue oxygen delivery. Science 340, 1327–1329.
Santovito, G., Marino, S.M., Sattin, G., Cappellini, R., Bubacco, L., Beltramini, M., 2012. Cloning and characterization of cytoplasmic carbonic anhydrase from gills of four Antarctic fish: insights into the evolution of fish carbonic anhydrase and cold adaptation. Polar Biol. 35, 1587–1600.
Shu, J.J., Harter, T.S., Morrison, P.R., Brauner, C.J., 2018. Enhanced hemoglobin–oxygen unloading in migratory salmonids. J. Comp. Physiol. B. 188, 409–419.
Supuran, C.T., 2008. Carbonic anhydrases-an overview. Curr. Pharm. Des. 14, 603–614.
Tashian, R.E., 1989. The carbonic anhydrases: widening perspectives on their evolution, expression and function. Bioessays 10, 186–192.
Tufts, B., Esbaugh, A., Lund, S., 2003. Comparative physiology and molecular evolution of carbonic anhydrase in the erythrocytes of early vertebrates. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 136, 259–269.
Wang, Y., Henry, R.P., Wright, P.M., Heigenhauser, G.J., Wood, C.M., 1998. Respiratory and metabolic functions of carbonic anhydrase in exercised white muscle of trout. Am. J. Physiol. Reg. Integr. Comp. Physiol. 275, R1766–R1779.
Wang, Z., Ying, Z., Bosy-Westphal, A., Zhang, J., Schautz, B., Later, W., Heymsfield, S.B., Müller, M.J., 2010. Specific metabolic rates of major organs and tissues across adulthood: evaluation by mechanistic model of resting energy expenditure. Am. J. Clin. Nutr. 92, 1369–1377.
Watson, C.J., Nordi, W.M., Esbaugh, A.J., 2014. Osmoregulation and branchial plasticity after acute freshwater transfer in red drum, *Sciaenops ocellatus*. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 178, 82–89.
Wright, P.A., Wood, C.M., 2009. A new paradigm for ammonia excretion in aquatic animals: role of Rhesus (Rh) glycoproteins. J. Exp. Biol. 212, 2303–2312.
Würtz, J., Salvenmoser, W., Pelster, B., 1999. Localization of carbonic anhydrase in swimbladder of European eel (*Anguilla anguilla*) and perch (*Perca fluviatilis*). Acta Physiol. 165, 219–224.