A cytosolic carbonic anhydrase molecular switch occurs in the gills of metamorphic sea lamprey

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Carbonic anhydrase plays a key role in CO₂ transport, acid-base and ion regulation and metabolic processes in vertebrates. While several carbonic anhydrase isoforms have been identified in numerous vertebrate species, basal lineages such as the cyclostomes have remained largely unexamined. Here we investigate the repertoire of cytoplasmic carbonic anhydrases in the sea lamprey (Petromyzon marinus), that has a complex life history marked by a dramatic metamorphosis from a benthic filter-feeding ammocoete larvae into a parasitic juvenile which migrates from freshwater to seawater. We have identified a novel carbonic anhydrase gene (ca19) beyond the single carbonic anhydrase gene (ca18) that was known previously. Phylogenetic analysis and synteny studies suggest that both carbonic anhydrase genes form one or two independent gene lineages and are most likely duplicates retained uniquely in cyclostomes. Quantitative PCR of ca19 and ca18 and protein expression in gill across metamorphosis show that the ca19 levels are highest in ammocoetes and decrease during metamorphosis while ca18 shows the opposite pattern with the highest levels in post-metamorphic juveniles. We propose that a unique molecular switch occurs during lamprey metamorphosis resulting in distinct gill carbonic anhydrases reflecting the contrasting life modes and habitats of these life-history stages.

The sea lamprey, Petromyzon marinus, Linnaeus 1758 is a basal vertebrate characterized by a complex anadromous life cycle. The larvae or ammocoetes are benthic, freshwater filter feeders that undergo a dramatic morphological and physiological transformation into parasitic feeders that migrate downstream to the sea. At the end of the marine trophic phase adults re-enter fresh water and migrate upstream to spawn and then die1,2.

Carbonic anhydrase is a zinc metalloenzyme, primarily involved in the reversible hydration/dehydration reactions with CO₂, thus involved in CO₂ transport and ionic and acid–base regulation3. Although carbonic anhydrases are found in all animals, in vertebrates only the α-carbonic anhydrase family is present4–6. In mammals, carbonic anhydrases are categorized according to their subcellular localization. Carbonic anhydrase isoforms 1, 2, 3, 7 and 13 form the functional cytosolic group7 and CA5 orthologues are mitochondrial8. The remaining carbonic anhydrase isoforms are membrane-associated with an extracellular orientation (4, 9, 12, 14, 15), secreted (6) or non-catalytic (8, 10, 11) (reviewed by Gilmour and Perry9).

The teleost cytosolic carbonic anhydrase gene repertoire is notably different from that of mammals9. Phylogenetic analyses indicate that teleosts retained an ancestral state of a single high activity carbonic anhydrase isoform, in contrast with the carbonic anhydrase gene expansion and functional segmentation in mammals8 and we have renamed this clade as ca17. In teleost fishes the ca17b (blood type; ca2-like b or ca2b) has been found to be mainly expressed in red blood cells (RBC) in zebrafish10 and trout11,12. In contrast, ca17a (cac or ca2-like a) has higher expression in gill than kidney and RBC10,12. More recently, cytoplasmic carbonic anhydrases have been cloned and characterized from gills of the Antarctic fishes Trematomus eulepidotus, Trematomus lepidorhinius, Trematomus bernacchii and Cygnodraco mawsoni13. Cytosolic carbonic anhydrases have also been cloned

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from blood samples of various non-teleost fishes such as the holostean gar, *Lepisosteus osseus*14, the elasmobranch dogfish, *Squalus acanthias*15, and from cyclostomes such as lamprey, *P. marinus*16, and the Pacific hagfish, *Eptatretus stoutii*17. To date only a single carbonic anhydrate isoform has been characterized in sea lamprey gill tissues and blood16.

For acid-base regulation, fishes rely on metabolic compensation, which involves the exchange of acid-base equivalents directly from their external environment: H+ and HCO3− are exchanged for Na+ and Cl−, respectively. Cytosolic carbonic anhydrase is an essential component of this process, providing an intracellular pool of H+ and HCO3− from CO2 hydration for these exchange processes in ion and acid-base regulatory epithelia such as the gill and kidney9. Although RBC carbonic anhydrase is central to transport of CO2 as HCO3− in the plasma in most vertebrates, the lack of functionally significant Cl−/HCO3− exchange (band 3 protein) in lamprey RBCs limits transport to intracellular RBC HCO3−16.

In the present study, we tested the hypothesis that metamorphosis, which marks a dramatic change in the life style and physiology of sea lamprey, requires changes in cytosolic carbonic anhydrate expression. A similar shift has already been documented in amphibian development and has been postulated to be related to distinct life stage strategies and physiological challenges (see review from Tufts et al.18). To address the hypothesis we cloned and sequenced a novel ammocoete carbonic anhydrate orthologue (Ca19) and together with the previously described carbonic anhydrate (ca18)16 elucidated changes during metamorphosis. Expression levels of the carbonic anhydrate lamprey genes were determined at the transcript level using quantitative RT-PCR, and protein level using immunoblotting and further characterized by MALDI-TOF mass spectrometry and molecular modelling. Adaptive changes following salinity exposure that would occur following the normal downstream migration of sea lamprey were also explored.

**Results**

**Diversity and evolution of cytosolic CA isofoms in vertebrates.** We began by examining the repertoire of carbonic anhydrate sequences in the sea lamprey genome (www.ensembl.org, Pmarinus_7.0). Following that, through PCR we were able to identify a previously unreported carbonic anhydrate gene in this species. The novel carbonic anhydrate sequence was identified in the ammocoete gill. The new transcript is 1786 bp with an ORF of 1592 bp (771 bp of coding region, 3′ UTR of 451 bp). The novel carbonic anhydrate has an AA identity with the published published carbonic anhydrate isoform from lamprey and 55.9% to 57.4% and 51.3% to 57.8% with rainbow trout and human Cac (Ca17a) and CA2 isoforms, respectively (Supplemental Fig. 1). Following identification of the novel carbonic anhydrate sequence in the lamprey genome it was not included in the present analysis (see materials and methods). The aa were aligned using ClustalW (BioEdit 7.0.9.0). Numbers represent the location of each amino acid relative to alignment with human CA2.

|                  | 1       | 2       | 3       | 4       | 5       | 6       | 7       | 8       | 9       | 10      | 11      | 12      | 13      | 14      | 15      | 16      | 17      | 18      |
|------------------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|---------|
| **Hsa CA2**      | Y       | S       | N       | N       | H       | S       | F       | N       | E       | I       | Q       | H       | H       | E       | H       | E       | H       | V       |
| **Pma Ca19**     | .       | .       | S       | .       | D       | G       | .       | .       | .       | .       | .       | .       | .       | .       | .       | .       | .       | .       |
| **Pma Ca18**     | .       | .       | .       | S       | K       | .       | .       | .       | .       | .       | .       | .       | .       | .       | .       | .       | .       | .       |
| **Omy Ca17a**    | .       | .       | .       | Q       | T       | K       | .       | .       | .       | .       | .       | .       | .       | .       | .       | .       | .       | .       |
| **Hsa CA1**      | .       | .       | V       | .       | H       | N       | F       | .       | .       | .       | .       | .       | .       | A       | L       | .       | .       | .       |

**Table 1.** Comparative analysis of carbonic anhydrate putative active site pocket amino acid residues and amino acids whose side chains either project into or border the active site in lamprey (Pma) Ca18 (GenBank: AAZ83742.1) and Ca19 (GenBank: ALM25804.1), with rainbow trout (Omy) Cac (Ca17a) (GenBank, NP_001166020.1), and human (Hsa) CA1 (GenBank, NP_001729.1) and 2 (GenBank, NP_0000583.1) modelled after Tashian et al.4 and Gilmour et al.15. The aa were aligned using ClustalW (BioEdit 7.0.9.0). Numbers represent the location of each amino acid relative to alignment with human CA2.

*Active site aa residues; Z, zinc binding ligand; +, proton shuttling associated ligand; ~, substrate associated pocket.*
Figure 1. Maximum likelihood phylogenetic tree describing relationships among carbonic anhydrase proteins from representative vertebrate taxa. Node values represent branch support using a Bayes algorithm (values below 0.5 are not shown). Asterisk indicates zfin.org or ensemble.org name. Accession numbers for all sequences are provided in the supplementary material.
and CA1/2/3/13/17 (Fig. 1). Based on this analysis we rename the sea lamprey carbonic anhydrase described by Esbaugh and Tufts\textsuperscript{16} ca18 and the novel sequence carbonic anhydrase ca19. The present analysis also provides some insight into the duplication timing and origin of CA1/2/3/13/17 isoforms. Despite the poor statistical support in some internal nodes within the CA1/2/3/13/17 clade, the combination with genome mapping information of these genes in various gnathostome lineages, allows us to propose that the expansion of the CA1/2/3/13 clade took place after the divergence of coelacanth from tetrapods (Fig. 1; Supplemental Fig. 2). Thus, ca17 “one-to-one” orthologues are found in chondrichyans, coelacanth and gars with two teleost-specific genome duplicates, ca17a and ca17b. Moreover, we find a further carbonic anhydrase gene unique to amphibians, named ca2b, and an additional CA3 in birds, named CA3b.

The finding that the two lamprey cytosolic isoforms group together and are at the base of the gnathostome CA1/2/3/13/17 and CA7 clades complicates a conclusion over their true orthology. To provide further insights into their origin, we examined the genomic locations of the lamprey carbonic anhydrase genes and compared them with those of their human counterparts (Supplemental Fig. 3). However, this information is available for the lamprey ca5 and novel cytosolic carbonic anhydrase but not the previously described cytosolic carbonic anhydrase\textsuperscript{16}. The human cytosolic carbonic anhydrase genes localize to genomic regions related by genome duplication, the so-called 2 R, at linkage group 3\textsuperscript{2}. In the case of the lamprey, we find that the ortholog of the gene close to ca5 (heatr3) maps to human chromosome 16 (Supplemental Fig. 3), providing strong support of its orthology. In contrast, the human orthologues of the genes in the vicinity of the lamprey novel cytosolic carbonic anhydrase do not localize to either chromosome 8 or 16 as would be expected if this gene was a true ortholog of either CA1/2/3/13 or CA7, respectively. However, we find some clues that indicate that the lamprey novel cytosolic carbonic anhydrase might represent a retained paralogue resulting from 2 R, but subsequently lost in gnathostomes. In effect, two genes, clc4f119 and a novel clc gene indicate that this genomic region in lamprey is probably orthologous of a region of the human genome in chromosome 14 which is paralogous to the regions in chromosomes 8 and 16 (Supplemental Fig. 3). Considering these observations and the phylogenetic results, we suggest that the lamprey cytosolic carbonic anhydrases might represent novel carbonic anhydrase gene lineages resulting from genome duplications in vertebrate ancestry, which have been retained uniquely in the cyclostomes lineage. We thus propose calling the previously described\textsuperscript{16} and novel cytosolic carbonic anhydrases ca18 and ca19, respectively, in line with the naming of new carbonic anhydrases. The recent proposal of a single genome duplication in the vertebrate ancestor would imply a different interpretation of our data\textsuperscript{22}. Thus, additional mapping and phylogenetic data from other cyclostome species should help to clarify these issues.

**Tissue distribution.** At the mRNA level, the cytosolic carbonic anhydrase isoform ca19 was expressed in ammocoete gill, blood, and gut (anterior and posterior intestine) at similar levels with significantly lower expression in the kidney (Fig. 2a). In post-metamorphic juveniles, RBC had relative higher mRNA expression than the other tissues tested but ca19 levels were otherwise significantly lower than in any ammocoete tissue tested. The mRNA expression of ca18 was significantly higher in all post-metamorphic lamprey tissues except kidney (Fig. 2b). Blood had the highest mRNA expression levels of ca18 in post-metamorphic lamprey with no significant differences between the other tissues. In ammocoetes, ca18 mRNA expression was significantly higher in kidney, with intermediate expression in gill and blood and the lowest expression in gut (anterior and posterior intestine). RBC's contamination in each type of tissue was assessed by the analysis of the corresponding mRNA expression of hemoglobin in tissue samples. Lamprey hemoglobin hba2a (\textasciitilde aHb2a) showed the highest mRNA expression levels in post-metamorphic juvenile RBC's with similar lower levels (\textasciitilde 1%) in the other tissues (Fig. 2d). In ammocoetes, mRNA expression levels of hba2a were significantly lower than in postmetamorphic juveniles. In contrast, in ammocoetes the putative larval hemoglobin hba9 (\textasciitilde aHb9) mRNA levels were significantly higher in blood (>50-fold) compared to all other tissues (Fig. 2c), indicating that blood was unlikely to contribute to observed differences in tissue carbonic anhydrase mRNA levels. The hba9 mRNA levels were also significantly lower in post-metamorphic juveniles than in ammocoetes (Fig. 2e). In addition, ca19 and ca18 expression were calculated using hba9 and hba2a as respective reference genes and tissue mRNA levels expressed relative to RBC groups to further assess RBC contamination (Fig. 2f, respectively). In ammocoetes, the gill and gut (anterior and posterior intestine) ca19 was significantly higher than RBC (and kidney). In post-metamorphic lamprey, ca18 levels were significantly higher in gill and anterior intestine only. The mRNA expression levels of the reference genes gapdh and 18s were found not to be consistent across the tissues tested (Supplemental Fig. 4).

**Changes in cytosolic carbonic anhydrase mRNA levels during metamorphosis and increasing salinity acclimation of post-metamorphic juveniles.** Higher branchial transcript levels of the novel ca19 isoform were found in ammocoetes compared to post-metamorphic juveniles and adults. Relative mRNA expression levels of sea lamprey ca18 and ca19 isoforms in gill tissue were assessed using real time RT-PCR (Fig. 3a,b). The analyses indicated that the ca19 isoform is almost exclusively expressed in the ammocoete and during the 1st and 2nd stages of metamorphosis. Low levels of expression of ca18 were found during these stages, which increased significantly from stage 5 onward during metamorphosis. Post-metamorphic juveniles show an increase of ca18 mRNA expression in response to increased salinity (Supplemental Fig. 5).

**Immunoblotting and 2-DE analysis.** Probing of lamprey gill immunoblots with a heterologous mammalian CA2 antibody revealed the presence of two immunoreactive bands at 27 and 29 kDa, respectively (Fig. 4). A significant difference in band expression was found in ammocoetes, metamorphic stages 1 to 2 and juveniles. Expression of the 27 kDa band was higher in ammocoetes and stages 1–2 and decreased significantly during metamorphosis and was undetectable in post-metamorphic juveniles. In contrast the 29 kDa protein band expression was significantly lower in ammocoetes and stages 1–2 and increased significantly at the latter stages of metamorphosis (stage 5–7, post-metamorphic juvenile). Consequently, protein expression of the 27 kDa band was...
significantly greater than the 29 kDa band in ammocoetes and early metamorphic stages while no significant differences were found in protein band expression for stages 5 to 7. In post-metamorphic juveniles protein expression of the 29 kDa band was significantly greater than the 27 kDa band. To confirm the identity of the two bands, a proteomics approach was taken. The 2-DE gel immunoblots probed with the heterologous cytosolic carbonic anhydrase antibody (Supplemental Fig. 7) indicated spots of potential interest. The 2-DE gel spots were examined by MS/MS and database search (Supplemental Fig. 6, Table 5). High protein identification scores revealed the presence of two Ca18 (Ca18-i, Ca18-ii) in RBC’s of ammocoetes and post-metamorphic lamprey (Supplemental Fig. 6A) and six Ca19 spots (Ca19-i, Ca19-ii, Ca19-iii, Ca19-iv, Ca19-v and Ca19-vi) in the RBC’s of ammocoetes only (Supplemental Fig. 6B). In agreement with the 1D western blots, the six Ca19 spots corresponded to the 27 kDa band and the Ca18 spots to the larger 29 kDa band. The theoretical pI values for Ca18 and Ca19 were estimated at 5.51 and 6.51, respectively, with a prediction of 20 and 19 probable modifications by phosphorylation each (ProMoST23, Supplemental Table 7), with predicted pI values ranging from 4.64–5.39 and 5.05–6.27. The NetPhos 3.1 Server analysis predicted 27 and 34 potential phosphorylation sites for Ca18 and Ca19, respectively (Supplemental Table 8; Supplemental Fig. 1). The respective non-phosphorylated pI values using Compute pI/Mw tool were 5.47 and 6.23.

**Discussion**

We have identified and characterized a novel cytosolic carbonic anhydrase isoform, *ca19*, which is highly expressed in the gill and RBCs of sea lamprey during its early life history stages. In addition we have compared significantly greater than the 29 kDa band in ammocoetes and early metamorphic stages while no significant differences were found in protein band expression for stages 5 to 7. In post-metamorphic juveniles protein expression of the 29 kDa band was significantly greater than the 27 kDa band. To confirm the identity of the two bands, a proteomics approach was taken. The 2-DE gel immunoblots probed with the heterologous cytosolic carbonic anhydrase antibody (Supplemental Fig. 7) indicated spots of potential interest. The 2-DE gel spots were examined by MS/MS and database search (Supplemental Fig. 6, Table 5). High protein identification scores revealed the presence of two Ca18 (Ca18-i, Ca18-ii) in RBC’s of ammocoetes and post-metamorphic lamprey (Supplemental Fig. 6A) and six Ca19 spots (Ca19-i, Ca19-ii, Ca19-iii, Ca19-iv, Ca19-v and Ca19-vi) in the RBC’s of ammocoetes only (Supplemental Fig. 6B). In agreement with the 1D western blots, the six Ca19 spots corresponded to the 27 kDa band and the Ca18 spots to the larger 29 kDa band. The theoretical pI values for Ca18 and Ca19 were estimated at 5.51 and 6.51, respectively, with a prediction of 20 and 19 probable modifications by phosphorylation each (ProMoST23, Supplemental Table 7), with predicted pI values ranging from 4.64–5.39 and 5.05–6.27. The NetPhos 3.1 Server analysis predicted 27 and 34 potential phosphorylation sites for Ca18 and Ca19, respectively (Supplemental Table 8; Supplemental Fig. 1). The respective non-phosphorylated pI values using Compute pI/Mw tool were 5.47 and 6.23.

**Discussion**

We have identified and characterized a novel cytosolic carbonic anhydrase isoform, *ca19*, which is highly expressed in the gill and RBCs of sea lamprey during its early life history stages. In addition we have compared
this novel isoform with the cytosolic carbonic anhydrase (ca18) previously described in adult sea lamprey by 
Esbaygh and Tufts16. Our findings indicate a clear and sustained isoform switch during metamorphosis. The 
ca19 isoform is expressed during the ammocoete stage and during the initial stages of metamorphosis. In contrast, 
ca18 mRNA and protein is expressed at low levels during the larval stage and becomes more highly expressed 
only during the latter stages of metamorphosis and into the post-metamorphic stages when the sea lamprey is 
prepared to enter its marine trophic phase2. These results suggest ca19 isoform may play an important role during 
the time sea lamprey live in the silty substrates of freshwater streams when the ammocoete is hyperosmoregulat-
ing and lives as a filter feeder on a low energy diet.

The carbonic anhydrase gene family has a wide diversity of isoforms and functions among fish groups9. 
However, our understanding of carbonic anhydrase diversity, function and distribution is still incomplete when 
considered in an evolutionary framework. Prior to this study a single high-activity cytosolic carbonic anhydrase

Figure 3. Relative mRNA expression (mean ± S.E.M.) of P. marinus ca18 and ca19 in ammocoete (A; N = 16), 
metamorphosis stages 1 to 2 (N = 12), 5 to 6 (N = 5), 7 (N = 3) and post-metamorphic (T; N = 8). Cropped 
representative bands from qPCR reactions from the same run are shown above. Changes in ca18 and ca19 are 
analyzed separately and bars with like letters are not significantly different from each other (in lower and upper 
figure case letters, respectively). Two-way ANOVA and SNK post-hoc test P < 0.05.

Figure 4. Representative cropped western blot of sea lamprey gill carbonic anhydrase expression 
(mean ± S.E.M.) using a heterologous cytosolic carbonic anhydrase antibody (1:2000) with crossreactive 
27 kD and 29 kD bands in a developmental stage specific pattern collected under identical experimental 
conditions. The developmental series includes ammocoete (N = 10); metamorphic stages 1 to 2 (N = 20); 
stages 5 to 7 (N = 7); and post-metamorphic juvenile (N = 15). Changes in 27 kD and 29 kD bands are analyzed 
separately and bars with like letters are not significantly different from each other (in upper and lower case letters, respectively). Significant differences between 27 kD and 29 kD bands within a developmental group are indicated by an asterisk. See supplemental Fig. 8 for original blots. Two-way ANOVA and SNK post-hoc test 
P < 0.05.
isoform (ca18) had been found in the sea lamprey, although the evolutionary relationships to other vertebrate carbonic anhydrases is contentious\(^{13,16}\). This isoform was found in a variety of tissues including blood, brain, kidney and gill but absent in muscle, liver and intestine in adult lamprey using Northern blotting\(^{28}\). In our study we have identified a novel cytosolic carbonic anhydrase, isoform 19, which is preferentially expressed in the lamprey’s larval stages in tissues such as gill, blood and anterior and posterior intestine. The discrepancy in intestinal expression between the two studies can be explained by the differences in the life history stages and the sensitivity of the techniques that were used. Esbaugh and Tufts\(^{16}\) studied adults on their upstream spawning migration, and the digestive system is known to degenerate as lampreys do not feed at this stage and die after spawning\(^{24}\). Northern blotting is also less sensitive than PCR based techniques used in the present study\(^{25,26}\). In contrast, our findings of ca18 and ca19 mRNA expression indicate the presence of cytoplasmic carbonic anhydrases in the gut during larval and post metamorphic stages, when the digestive system is fully functional. In addition, the hematopoietic tissue in larval lamprey is in the gut associated typhosol\(^{27,28}\) and thus high carbonic anhydrase mRNA expression would be expected\(^{29}\). As for aHb expression, which we used as an indicator of RBC contamination of tissues, our results indicate a switch from aHb9 to aHb2a in the ontogeny of sea lamprey in agreement with a recent report\(^{30}\).

The molecular mass of Ca19 determined in silico closely matched the values measured experimentally by gel electrophoresis. This supports our findings indicating changes in protein levels through immunoblotting from gill tissue probed with a heterologous CA2 antibody. The 27 kD band shows higher relative protein levels during earlier life-history stages, while during metamorphosis there is a shift in the protein expression of both bands. In the final metamorphic stages the 29 kD band is much more intense while the 27 kD band decreases and is undetected in fully transformed lampreys. Notably, the predicted molecular masses of Ca19 and Ca18 correspond to the 27 and 29 kDa bands, respectively. However, in order to confirm the reactivity of both isoforms with the heterologous CA2 antibody we utilized an MS/MS analysis approach. This approach confirmed a match with the previously published carbonic anhydrase sequence (Ca18) by Esbaugh and Tufts\(^{16}\) in the RBC’s of post-metamorphic lamprey and the carbonic anhydrase sequence identified with this work (Ca19) in RBC’s of ammocoetes. The pl of eukaryotic proteins provides insight into their subcellular localization\(^{31}\) and experimental pl can be applied to distinguish protein isoforms and modifications\(^{32}\).

In most cases the pl predicted by the databases closely matches the experimentally determined value\(^{23-35}\), nevertheless it is not uncommon to experimentally observe shifts in these value. These shifts are due to protein modifications such as truncations and deletions but are more often associated with co- or post-translational phosphorylation. Phosphorylation plays a key role in regulatory mechanisms in cells\(^{36-38}\) by replacing neutral hydroxyl groups on serine, threonine or tyrosine residues with phosphate group(s) that are negatively charged\(^{39}\). As a result, phosphorylation typically induces an acidic shift in pl. In agreement, analysis using ProMoST demonstrated that all experimental pl’s were found lower than their respective nonphosphorylated theoretical pl value. In addition to phosphorylation, the multiple Ca18 and Ca19 protein spots in this study may result from pl shifts attributed to acetylation at the N-terminal of the residues sequence. This has already been documented for acidic and neutral protein\(^{35}\), where the removal of amino group(s) by this process results in an adjustment of the acid-base balance and shifts the protein pl value.

Previous phylogenetic analyses that have shown that fish cytoplasmic carbonic anhydrase diverged prior to the gene duplication events that gave rise to the mammalian carbonic anhydrase gene cluster\(^{11,12,14}\) with the exception of CA7, where orthologues have also been found in fish\(^{40}\). In mammals both high and low turnover carbonic anhydrase isoforms can be found while only high turn over isoforms are present in fish and are catalytically akin to mammalian CA2\(^{40}\). However, we were able to identify a new cytoplasmic carbonic anhydrase in lamprey. Our findings suggest that cyclostomes retain a unique cytoplasmic carbonic anhydrase set, since both phylogenetics and synteny analysis indicate that ca18 and ca19 might not be orthologues of previously described carbonic anhydrase genes in gnathostomes. The examination of the carbonic anhydrase genomic loci genes in lamprey and humans supports the hypothesis that duplications in vertebrate ancestry were instrumental in the elaboration of carbonic anhydrase diversity. We hypothesize that the cyclostomes’ cytoplasmic carbonic anhydrase genes probably represent retained genome duplicate paralogues. In effect, the lamprey carbonic anhydrase genes might represent genome duplicates of a different origin to gnathostomes. It has recently been put forward that vertebrates underwent one genome duplication, in contrast to the two or three rounds previously proposed\(^{20,41}\), which does not allow us to determine the exact duplication event from which cyclostome ca18 and ca19 arose.

An analysis of the Ca19 active site pocket revealed seven and eight amino acid differences from Ca18\(^{16}\), and rainbow trout Ca17a (cac)\(^{11,12,19,42,43}\), respectively, both of which have high catalytic rates. In comparisons with the mammalian low turnover CA1 and high turnover CA2, the Ca19 isoform shows twelve and nine amino acid differences from their respective active site pockets. However, for the most part the amino acid differences are predicted to be substitution neutral, and significantly the essential proton shuttle histidine-64 (H) and zinc binding ligand residues (H94, H96, H119) are conserved. However, two of the amino acids that border or have side chains projecting into the active site at positions 91 and 204 show predicted unfavourable substitution ratings (−2 and −3, respectively). At residue 91 conformationally flexible glycine (G) is found in place of hydrophobic isoleucine (I), and at residue 204 polar asparagine (N) is found in place of hydrophobic leucine (L). These and the other neutral predicted amino acid differences might impart changes in the three dimensional structure\(^{44}\) and access to the catalytic site potentially impacting Ca19 activity, kinetics and/or inhibitor binding\(^{45}\). Analysis using NetPhos 3.1 Server demonstrated that five possible phosphorylation sites correspond to active site pockets of Ca19 (tyrosine-7 (Y), serine-29 (S), S67, threonine-200 (T) and T244 represented in Table 1), which are likely to alter the catalytic activity of the enzyme as demonstrated in rainbow trout by Carrie and Gilmour\(^{46}\).

In our analysis of the electrostatic potential of cytosolic carbonic anhydrases in fishes, we find that RBC carbonic anhydrases have values around 1.0 with the exception of zebrafish Ca17b (cahz), whereas tissue carbonic anhydrases have negative electrostatic potentials that tend to be more variable. Marino et al.\(^{45}\) observed that both C. hamatus and trout tissue type Ca17a shared a similar negative electrostatic potential in contrast to RBC Ca17b.
carbonic anhydrases suggesting a diversification of fish isoforms based more on cell type than species. These differences may reflect the dominate roles of RBC and tissue carbonic anhydrases in blood CO₂ transport, and ion and acid–base regulation, respectively, related to protein interactions. Although both lamprey Ca18 and Ca19 have been demonstrated to be expressed in RBC, they both have negative electrostatic potential values which may reflect the different mode by which lamprey RBCs participate in the convective transport of blood gases.¹⁸

Cytosolic carbonic anhydrases in the gill are important for whole animal ion and acid-base regulation providing an intracellular pool of H⁺ and HCO₃⁻ from CO₂ hydration for exchange with Na⁺ and Cl⁻, respectively, as well as for aiding in metabolic processes and acid-base regulation of individual gill cells that have a high metabolism and generate excessive CO₂ levels. Branchial cytosolic carbonic anhydrases apparently do not have a role in facilitating respiratory CO₂ elimination.³⁷ We propose that loss of the novel ammocoete isoform ca19 is associated with the loss of the enigmatic ammocoete mitochondrion-rich cells (MRC) and that the increase in ca18 is triggered by metamorphosis as preparation for the higher activity marine trophic phase of this species’ lifecycle.

In ammocoetes, Conley and Mallatt⁴⁷ have localized carbonic anhydrase activity by enzyme histochemistry to the lamellar epithelium and RBCs. Significantly, this lamellar epithelial localization of carbonic anhydrase corresponds to the location of the ammocoete MRCs which are unique to the ammocoete stage and are lost during metamorphosis.⁵⁻⁶⁸ This pattern of loss mirrors that of ca19. The ammocoete MRCs make up ~60% of epithelial cells covering the gill lamellae and are mitochondrion-rich. The function of ammocoete MRCs is unknown although various hypotheses have been presented that include ion uptake and metabolic waste elimination. The high mitochondrial density of these cells would indicate a high metabolic activity and cytosolic carbonic anhydrase would have a role in modulating cellular acid-base demands.¹ A role in ion regulation seems unlikely since Bartels and co-workers⁴⁹ found no morphometric changes with ion poor water challenges and these cells are not present in adult freshwater migrants which also need to hyperosmoregulate. In addition to the hyperosmotic gill epithelium, the ammocoete gill has very low NKA activity and neither NKA nor V-ATPase co-localize to these cells.⁵⁰ Instead it is the intercalated mitochondria-rich cells (IMRC) that express these ATPase that are predicted to drive ion regulatory processes.⁵⁰,⁵¹

In addition, we postulate that the environmental conditions ammocoetes live under may shape their carbonic anhydrase expression profile. Ammocoetes live buried in the silt and muddy substrate,²,³ where higher CO₂ levels and humic substances contribute to a more acidic environment.²² In substrate dwelling sand eel, Behrens et al.⁵³ have demonstrated that O₂ levels in the surrounding substrate drop and given that CO₂ is eliminated during the exhalation, it would be reasonable to expect that CO₂ levels would be higher. In trout, hypercapnia has been shown to increase branchial carbonic anhydrase (activity, protein and mRNA)⁴⁸⁻⁵⁰. While it is purely speculative at this point, the presence of ca19 during the ammocoete life stages might impart a higher CO₂ tolerance as an adaptation to this environment.

Branchial IMRC, which are expressed in other life history stages, are a more likely candidate for active ion uptake and acid-base regulation and we would propose as a site for the expression of ca18. Carbonic anhydrase immunoreactivity has been localized to IMRC using heterologous antibodies in freshwater post-metamorphic Geotria australis⁵⁳ and sea lamprey⁴⁰; however, in the latter study weaker immunoreactivity was also found throughout the rest of the gill epithelium. In both IHC studies, a similar apical localization with H⁺-ATPase was observed which supports the role of carbonic anhydrase as a provider of an intracellular supply of H⁺ for the pump through the carbonic anhydrase catalysed hydration of CO₂.⁴⁸,⁵⁷ In addition, results from the salinity acclimation of post metamorphic juveniles revealed an increase in ca18 mRNA expression suggesting that this isozyme plays an important role in adaptation to higher salinity environments as well. However, it should be noted that Reis-Santos et al.⁵⁰ did not observe a change in the pattern of CA-immunoreactive cells with salinity acclimation. Nonetheless, increasing expression of ca18 isoform at the CA-immunoreactive cells with salinity acclimation. Nonetheless, increasing expression of ca18 isoform at the
Germany). The fiducial limit was set at $P < 0.05$.

Sampling. Animals were killed with an overdose of ethyl-m-amino benzoate (MS-222 1:5000 buffered with sodium bicarbonate, pH 7.8 1:5000). Total length (mm), mass ($\pm 0.01$ g) and stage were recorded for each animal. Blood samples were collected from the caudal vessels using a heparinized capillary tube after caudal transection, centrifuged and hematocrit recorded. Separated plasma and RBCs were snap frozen in liquid nitrogen and stored at $-80^\circ$C. Gill, kidney, anterior intestine, and posterior intestine were excised and snap frozen in liquid nitrogen and stored at $-80^\circ$C for further use.

RNA isolation and PCR. Total RNA was isolated from tissue samples, quality assessed, quantified and converted to cDNA for PCR. Zebradish carbonic anhydrase primers were used to isolate an initial partial sequence that was completed by RACE. Real-time PCR was conducted using $ca18$ and $ca19$ specific primers. [See Supplemental Materials and Methods for more details].

Phylogenetic Analysis. Carbonic anhydrase sequences were collected from various genome databases such as Ensembl, GenBank or JGI (Joint Genome Institute) through Blastp searches. We also searched species-specific genome sites (e.g. http://esharkgenome.imcb.a-star.edu.sg/) to complete the screening of carbonic anhydrase gene diversity in vertebrates. Our analysis included all major vertebrate lineages with a total of 63 sequences (Accession numbers and Ensembl codes shown in the Supplemental Table 4). An ortholog of $CA5$ from lamprey was not included in the analysis because it caused long-branch attraction in the tree (not shown). Amino acid sequences were aligned using the MAFFT software with default parameters. The alignment was stripped of all columns containing gaps leaving 152 positions for phylogenetic analysis. A Maximum Likelihood tree was constructed at PhyML (http://www.atgc-montpellier.fr/phyml/) protein evolutionary model was calculated in PhyML using the smart model selection resulting in LG $+ I$. Branch support was estimated using the aBayes method as implemented in PhyML. Trees were visualized with FigTree (v1.4.2; http://tree.bio.ed.ac.uk/software/figtree/).

Immunoblotting. Gill tissue from the metamorphic series from ammocoete to juvenile stages were analyzed by immunoblotting as described in Reis-Santos and co-workers with modifications. Ten $\mu$g of sample were loaded onto polyacrylamide gels (10% T solving gels; 4% T stacking) and transferred to nitrocellulose membranes (Amersham (TM) Hybond (TM) ECL, GE Healthcare). Following blocking with 5% blotto, membranes were probed with a heterologous rabbit anti-bovine cytosolic CA polyclonal antibody ($1:2000$, Abcam Cambridge UK) or mouse anti-3-actin monoclonal (1:500; Sigma-Aldrich) overnight at room temperature. Membranes were then rinsed with TTBS (0.05% Tween-20 in Tris Buffered Saline, pH 7.4) and incubated for 1 hour with a goat anti-rabbit or anti-mouse IgG secondary antibodies conjugated to horseradish peroxidase, diluted in TTBS (1:50,000). Signal was obtained by enhanced chemiluminescence (ECL) with Millipore Immobilon Western chemiluminescent detection HRP substrate (Millipore Corporation, MA USA). Images were acquired using a luminescent image analyzer Fujifilm LAS-4000 mini and image reader software LAS-4000 version 2.0. Intensity of band signal was quantified using image analysis software Multi Gauge v3.1 (Fujifilm Tokyo, Japan). After detection, membranes were stripped with low pH stripping buffer and reprobed with other antibodies.

Two-dimensional electrophoresis (2DE), MALDI-TOF/TOF analysis, protein identification and modeling. Since both cytosolic carbonic anhydrase isoforms are expressed in RBCs and due to the ease of blood collection we performed proteomic analysis using RBCs. RBC samples (150 $\mu$L) from post-metamorphic juveniles and ammocoetes were prepared for two-dimensional electrophoresis followed by MALDI TOF/TOF analysis and protein identification as described in Campos and co-workers and homology modeling [See Supplemental Materials and Methods for more details]. Predicted phosphorylated forms of $Ca18$ and $Ca19$ were determined in silico using NetPhos 3.1 Server (http://www.cbs.dtu.dk/services/NetPhos/), ProMoST (http://prometheus.brc.mcw.edu/promost/) and Compute pi/Mw tool according to Gasteiger et al. (ExPaSy Server; http://web.expasy.org/compute_pi/).

Statistical analysis. Data are presented as means + standard error of the mean. Statistical differences in mRNA and protein expression between tissues and life stage groups were determined using two-way ANOVA followed by the post-hoc Student-Newman-Keuls (SNK) test. One way ANOVA and SNK tests were performed on ammocoete and juvenile $ca19$ and $ca18$ normalized with their respective hemoglobin genes and juveniles exposed to different salinities. The statistic program SigmaPlot 11.0 was used for all analyses (Systat Software, Inc., Germany). The fiducial limit was set at $P < 0.05$.

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Author Contributions

J.M.W., D.F.-M. and L.F.C.C. conceived the experiments, D.F.-M., S.D.M. and J.M.W. conducted the experiment(s), D.F.-M., L.F.C.C., M.L.-M., H.O., A.C. and S.D.M. analyzed the results. D.F.-M., J.M.W., L.F.C.C., M.L.-M., H.O., A.C., J.C. and S.D.M. reviewed the manuscript.

Additional Information

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