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Cytosolic carbonic anhydrase in the Gulf toadfish is important for tolerance to hypersalinity

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ABSTRACT

Carbonic anhydrase (CA) is a ubiquitous enzyme involved in acid–base regulation and osmoregulation. Many studies have demonstrated a role for this enzyme in fish osmoregulation in seawater as well as freshwater. However, to date CA responses of marine fish exposed to salinities exceeding seawater (~35 ppt) have not been examined. Consequently, the aim of the present study was to examine CA expression and activity in osmoregulatory tissues of the Gulf Toadfish, *Opsanus beta*, following transfer to 60 ppt. A gene coding, for CAc of 1827 bp with an open reading frame of 260 amino acids was cloned and showed high expression in all intestinal segments and gills. CAc showed higher expression in posterior intestine and rectum than in anterior and mid intestine and in gills of fish exposed to 60 ppt for up to 4 days. The enzymatic activity, in contrast, was higher in all examined tissues two weeks following transfer to 60 ppt. Comparing early expression and later activity levels of acclimated fish reveals a very different response to hypersalinity among tissues. Results highlight a key role of CAc in osmoregulation especially in distal regions of the intestine; moreover, CAc play a role in the gill in hypersaline environments possibly supporting elevated branchial acid extrusion seen under such conditions.

1. Introduction

Gulf toadfish (*Opsanus beta*) are seawater teleosts found in coastal habitats ranging from the Gulf of Mexico to northern South America. These environments are shallow water habitats often influenced by freshwater inputs from canals and groundwater sources as well as evaporation that create a near-shore environment with low, and fluctuating, salinity (Lirman and Cropper, 2003). Gulf toadfish are abundant in Florida Bay, where salinity has been reported to range from a minimum of 24.2 in November to a maximum of 41.8 in July ppt (Kelble et al., 2007) and tolerate salinities from 5 to 60 ppt under laboratory conditions (McDonald and Grosell, 2006). A number of studies have examined osmoregulation by toadfish. Salinity influences the need for salt absorption by the intestine, a process with pronounced impacts on acid–base balance due to differences in intestinal bicarbonate secretion (Grosell and Genz, 2006; Grosell and Taylor, 2007; Genz et al., 2008). A single study has examined the role of the kidney in maintaining osmotic and ionic balance (McDonald and Grosell, 2006) and early studies have addressed the role of gill (Evans, 1982). However, limited information is available about the involvement of carbonic anhydrase (CA) in the osmoregulatory process.

Carbonic anhydrase catalyzes the reversible hydration/dehydration reactions of CO_2, producing equivalent amounts of H^+ and HCO_3^- ($CO_2 + H_2O \rightarrow H^+ + HCO_3^-$). This enzyme is found in many different tissues and is involved in a number of different physiological processes, including bone formation, calcification, ion transport, acid–base balance, and carbon dioxide transport (Gilmour and Perry, 2009).

In freshwater fish, the gills are the principal site for Na^+ and Cl^- uptake to maintain ionic and osmotic balance. Acid–base regulation is coupled to ionic movement because acid–base compensation relies primarily on the direct transfer of H^+ and HCO_3^- in exchange for Na^+ and Cl^-, respectively across the gills (Sender et al., 1999; Claiborne et al., 2002; Marshall, 2002; Hirose et al., 2003; Perry et al., 2003; Evans et al., 2005; Gilmour and Perry, 2009). Carbonic anhydrase, abundantly present in gill epithelial cells, plays a role in these processes by catalysing the hydration of CO_2 to provide H^+ and HCO_3^- at a high rate (Perry and Laurent, 1990). In contrast, salt secretion by the gills in marine fish is not believed to be associated with CA, although the gill is an important site for regulation of acid–base balance (Marshall and Grosell, 2005).

In marine teleosts, the intestine is an important site for Na^+ and Cl^- transport to maintain osmotic balance. It is well documented that marine fish must drink seawater to compensate for continual water loss caused by their concentrated environment (Marshall and Grosell, 2005; Gilmour and Perry, 2009). Intestinal water absorption is driven by NaCl uptake. Absorption of Cl^- occurs in part by apical Cl^-/HCO_3^-

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exchange, with HCO_3^- provided by transepithelial transport and/or by CA-mediated hydration of endogenous epithelial CO_2 (Grosell, 2006; Grosell et al., 2009). Hydration of CO_2 also liberates H^+, which are eliminated mainly across the basolateral membrane of the intestinal epithelium (Grosell et al., 2001,2005; Genz et al., 2008). Previous studies on the gulf toadfish and seawater acclimated rainbow trout demonstrated that hydration of endogenous CO_2 induces apical secretion of HCO_3^-, and demonstrates the involvement of CAc and a membrane-bound, luminal CA IV isoform in intestinal HCO_3^- secretion (Grosell and Genz, 2006; Grosell et al., 2009).

Many studies report expression and activity of gill CA in fish acclimated at different salinities yielding contrasting results. In killifish (*Fundulus heteroclitus*) transferred from intermediate salinities to freshwater, expression of CA increased 12 h after transfer (Scott et al., 2005). In the gills of coho salmon (*Oncorhynchus kisutch*), CA activity was significantly higher in saltwater adapted fishes compared to freshwater fishes (Zbanyszek and Smith, 1984), while flounder (*Platichthys flesus*) showed no significant differences in CA levels between seawater and freshwater-adapted fish (Mashiter and Morgan, 1975; Sender et al., 1999). Similarly, no relationship to salinity was reported for CA activity in gills of the European eel (*Anguilla anguilla*; (Haswell et al., 1983)) and in *Tetraodon nigroviridis* the expression of gill CA was not significantly different between freshwater and saltwater acclimatized individuals (Tang and Lee, 2007). However, in agreement with the results from coho salmon, the specific CA activity in the gills of *Oreochromis mossambicus* increased with increasing salinity (Kültz et al., 1992).

Considering the intestine, expression or activity of CA increased two- to four-fold after seawater transfer in killifish (Blanchard and Grosell, 2006). In trout acclimated to 65% seawater the CA expression in intestine increased significantly after 24 and 48 h transfer and total CA activity was elevated after three weeks.

Few studies compare the CA expression and activity in gills and intestine. In *Dicentrarchus labrax* acclimated to salt- and freshwater, CA showed significantly higher expression levels in gills than in intestine in saltwater acclimated fish. In freshwater individuals, however, CA expression was not significantly different between tissues (Boutet et al., 2006). To our knowledge, CA expression and activity in osmoregulatory tissues of marine fish exposed to salinities exceeding normal seawater have never been examined. However, recent studies have demonstrated that other components of the pathways responsible for intestinal HCO_3^- secretion, which is elevated upon exposure to hypersalinity, are upregulated during exposure to elevated salinity (Genz et al., 2008; Taylor and Grosell, 2009). Furthermore, compensation for this increase in intestinal base excretion in the form of elevated branchial acid secretion has also been observed in the gulf toadfish following exposure to salinities exceeding those of normal seawater (Genz et al., 2008). Based on these observations we hypothesize that CA expression and activity will increase in these tissues following transfer to elevated salinity.

The aim of this project was to clone and sequence the toadfish cytoplasmic CA from intestinal tissue and to compare expression and activity of these CA isoforms in several different tissues (gill, anterior, middle and posterior intestine and rectum) of gulf toadfish (*Opsanus beta*) acclimated to control seawater (~40 ppt) and after transfer to 60 ppt.

2. Materials and methods

2.1. Experimental animals

Gulf toadfish (*Opsanus beta*, Goode and Bean, 1880) were obtained as by-catch from Biscayne Bay, FL, USA by shrimp fishermen and transferred to 62-L aquaria at the Rosenstiel School of Marine and Atmospheric Sciences in the summer of 2009. Immediately after transport, fish received a prophylactic treatment to remove ectoparasites (McDonald et al., 2003). The tanks received a continuous flow of filtered seawater from Biscayne Bay (specific locality: Bear Cut, 34–40 ppt, 22–26 °C) for at least one week before experimentation. Segments of polyvinyl chloride tubing were provided for shelter and the fish were fed pieces of squid to satiation twice weekly with food withheld for at least 48 h prior to sampling. Fish were maintained in the lab and used according to an IACUC-approved University of Miami animal care protocol.

2.2. Cloning of toadfish cytoplasmic carbonic anhydrase (CAc)

2.2.1. Total RNA extraction and cDNA synthesis

Fish were euthanized (tricaine methanesulfonate, MS-222; 0.2 g L^{-1}) and gills, sectioned intestine, and rectum were quickly placed into cryotubes and snap-frozen in liquid nitrogen. Extraction of total RNA was performed using RNA STAT-60 solution (Tel Test). Subsequently the RNA was DNase I treated using TURBO DNA-*free*™ (Ambion®) followed by gel electrophoresis to confirm that the integrity of RNA was maintained. Reverse transcription was performed with Invitrogen Superscript II Reverse Transcriptase using 1 µg of DNase I treated RNA and random hexamers.

2.2.2. 5′ and 3′ RACE

To obtain toadfish sequence for RACE primer design, an initial fragment (331 bp) of CAc was obtained by PCR of toadfish intestine cDNA. For this, degenerate primers were derived from alignment of multiple CAc sequences available in the Swiss-Prot database: *Danio rerio* (NM_199215.1), *Oncorhynchus mykiss* (NM_001124221.1), and *Tribolodon hakonensis* (AB055617.1). Reactions were performed using a MyCycler™ thermal Cycler (Bio-Rad) with Go-Taq Polymerase (Promega) (5 U µL^{-1}) using the following cycling parameters: 95 °C for 50 s, 56 °C for 50 s and 72 °C for 1 min. The primers used are shown in Table 1.

RACE reactions were carried out following the protocols provided with the BD SMART RACE cDNA Amplification Kit (BD Biosciences). Primers for RACE reactions (Table 1) were designed from a toadfish-specific sequence obtained from the degenerate PCR reaction. Touchdown PCR cycling conditions were as follows: five cycles of 94 °C for 30 s, 72 °C for 3 min followed by five cycles of 94 °C for 30 s, 68 °C for 30 s and 72 °C for 4 min followed by 25 cycles of 94 °C for 30 s, 68 °C for 30 s and 72 °C for 4 min. Products from all PCR reactions

Table 1
Primers used for qPCR and cloning of gulf toadfish (*Opsanus beta*) cytoplasmic carbonic anhydrase.

Primer	Sequence (5′–3′)
QFH (F)[a]	CAGTTYCAYTTCCAYTGGGG
QSPIDI (R)[a]	CAGTCTCCHATWGAYAT
ReCA (R)[a]	ACRATCCAGGTGACRCTCTC
Anchor (R)[a]	ACCACGCGTATCGATGTCG
RACE primer	
CA2-5p1-race	CCAGCAGAGGGGGCGTGGTCAGAGAGCC
CA2-5p2-race	ATCCATGTGACGCTCTCCAGCAGAGGG
CA2-3p1-race	CAGATCGGCGATAACCATGTCGGTC
CA2-3p2-race	GAGCTCCACCTGGTGCACTGGAACAC
EF1α primer	
EF1α-F	AGGTCATCATCCTGAACCAC
EF1α-R	GTTGTCCTCAAGCTTCTTGC
qPCR primer	
TFCA2-813F-qPCR	GGCCAAGTATCCCTGTGAGC
TFCA2-949R-qPCR	GAAGACCGACATGGTTATCGC
TFCA2-1136F-qPCR	TGGAGCAGATGGCCAAATTCC
TFCA2-1263R-qPCR	TCACTTGAAGGATGCACGGAC

[a] Primer sequences used for initial cloning of toadfish CAII fragment were designed from non conserved regions of fish CAII aligned sequences available in GenBank (see text for details).

were gel-purified with QIAquick® spin columns (Qiagen), TA-cloned into the pCR® 2.1-TOPO® vector (Invitrogen) and sequenced.

2.3. Molecular phylogenetic analysis

Phylogenetic studies were based on amino acid sequences available from EXPASY (http://www.expasy.ch/). Multiple alignments were performed using the Clustal W software (Thompson et al., 1994). The analyzed data set includes the following CA amino acid sequences: *Homo sapiens* CAI NP_001122303, II NP_000058, III NP_005172.1, IV BC069649, Va NP_001730, Vb NP_009151, VII AAH33865, IX AAH14950, XII BC023981, XIV AAH34412; *Mus musculus* CAI NP_001077426, II NP_033931, III NP_031632, IV NP_031633.1, Va NP_031634, Vb NP_851832 NP_062386, VII NP_444300 XP_134293, IX NP_647466.2, XII NM_178396, XIV AAH46995, XV AAK16671.1; *Gallus gallus* CAII P07630; *Xenopus tropicalis* CAII NP_001015729, XIV NM_001110051.1; *Petromyzon marinus* CA AAZ83742; *Danio rerio* CAII CAM13156, NP_954685, VII NP_957107 XP_692641; *Squalus acanthias* CAIV AAZ03744; *Oncorhynchus mykiss* CAI BAD36835, II NP_001117693, IV NP_001117959; *Lepisosteus osseus* CA AAM94169; *Tribolodon hakonensis* CA BAB83090; *Cyprinus carpio* CAII AAZ83743 and *Drosophila melanogaster* NP_648555 used as an outgroup.

Phylogenetic reconstructions were performed according to the Neighbor Joining (NJ) method (Saitou and Nei, 1987). In the former case, PAM substitution matrix (Dayhoff, 1978) was used in the reconstruction. Non parametric bootstrap resampling (BT) (Felsenstein, 1981) was performed to test the robustness of the tree topologies obtained. The tree topologies were visualized with the Treeview 1.6.6 program (Page, 1996).

2.4. Quantitative PCR (qPCR)

To assess the potential effect of transfer from seawater to 60 ppt water on expression of CAc in the gulf toadfish, cDNA templates, available from fish collected during the study of Grosell et al. (2009) at different times following the salinity challenge were subjected to quantitative PCR analysis. In addition, cDNA was prepared from perfused gills of fish kept in seawater and from fish maintained at 60 ppt for two weeks. PCR amplification and detection were performed in an MX4000 thermocycler (Stratagene). The reaction was carried out with AmpliTaq Gold polymerase (Applied Biosystems) and SYBR Green I (Sigma) as the fluorescent reporter dye. The amplification program consisted of one cycle of 95 °C for 5 min followed by 45 cycles at 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s. Samples from eight different fish were used for each tissue and for each different exposure time and salinity. Expression levels were calculated from log transformed cycle threshold (*CT*) values normalized to EF1α (tissue distribution) using a modification of the delta-delta-CT method (Livak and Schmittgen, 2001). *CT* values for *EF1α* did not change following transfer to 60 ppt.

2.5. Carbonic anhydrase activity

Ten fish (control) were held in a tank at 39 ± 2.0 ppt salinity, while an additional ten fish (experimental) were held in a tank at 60 ± 2.0 ppt salinity obtained by adding Instant Ocean to natural seawater. Both tanks were fitted with a recirculation filter and water was renewed every other day. The toadfish were fed frozen squid to satiation every other day for approximately two weeks prior to tissue isolation.

Fish were euthanized as above and gills were perfused with a heparinised NaCl saline via a PE10 cannula catheter inserted into the bulbus arteriosus. After the gills were cleared of red blood cells, filaments were obtained by dissection on ice. The mucosal lining of intestinal segments and the rectum were scraped off the underlying basement membrane and muscle layer using a microscope slide with the tissue placed with the muscle layer facing down on an inverted glass petri dish on ice. The tissues were placed in 500 μL buffer (10 mM TRIS, 225 mM mannitol, 75 mM sucrose, pH 7.4) and stored at −80 °C for later analysis. Tissues were thawed on ice and homogenized with an electronic tissue homogenizer (Ika t8 Ultra-Turrax), at maximum rate, in 1 mL buffer. Carbonic anhydrase (CA, EC 4.2.1.1 — carbonate dehydratase) activity was measured at 4 °C employing the ΔpH method as described in (Tufts et al., 1999) using a water jacketed reaction chamber of 2 mL and a Radiometer Analytical pHc 4000 combined pH electrode (Lyon, France) coupled to a Radiometer Analytical PHM 220 lab pH meter (Lyon, France). The pH meter was interfaced with a PC allowing for high resolution data collection. CA activity was normalized to total protein content of the sample determined by the Bradford assay (Bradford, 1976).

The CA activity was measured in the total tissue extract and in the cytosolic fraction. To obtain the cytosolic fraction the homogenate was centrifuged (100,000 × *g* for 90 min, L7-55 Beckmann ultracentrifuge; (Henry et al., 1993) at 4 °C to remove cellular debris, mitochondria and membrane fractions. The resulting supernatant was assumed to contain the cytosolic fraction.

2.6. Statistical analysis and data presentation

All data is presented as mean ± SEM calculated using SigmaPlot 3.0 (Jandel Scientific). Statistical analyses consisted of one-way or two-way analysis of variance (ANOVA) (OriginPro 8) followed by posthoc comparisons of individual means as appropriate. Means were considered significantly different at the level 0.05. Significant differences from control values are denoted in figures by an asterisk.

3. Results

3.1. Sequencing and phylogenetic analysis

The cloned cytoplasmic carbonic anhydrase (CAc) transcript from gulf toadfish (GenBank accession number GQ443599 is 1827 nucleotides long with an open reading frame that predicts a 260 amino acid protein. The translated sequence shows 78% identity to the CA of *Oncorhynchus mykiss* and *Pseudopleuronectes americanus*, 77–76% identity to other teleost CA amino acid sequences, and 63–59% identity to amphibian and mammalian orthologs. Phylogenetic analyses grouped the toadfish cytoplasmic CA sequence with other teleost sequences, and apart from tetrapod CA sequences and membrane associated CA sequences (CA IV, IX, XII, XIV, XV) (Fig. 1).

3.2. Carbonic anhydrase expression and activity

All intestinal segments and the rectal tissue showed similar CAc expression in seawater acclimated fish while the gills showed higher expression than the intestinal tissue (Fig. 2). High mean expression levels in response to exposure to elevated salinity were observed in the middle and posterior intestine and in the rectum (Fig. 3). It is worth noting that in Fig. 3, the values corresponding to 0 h (control) agree well with those from separate experiments reported in Fig. 2, in spite of the use of two different animal pools. The expression of elongation factor EF1α used to normalize CAc expression data remained constant, as evident from *CT* values (not shown), after salinity transfer, supporting the validity of our normalization. The middle intestine responded last with a higher expression at 96 h following transfer from seawater to 60 ppt, while the rectum CA expression had already increased at 12 h and remained stable and high through 96 h post transfer. The posterior intestine displayed a fast increase at 6 h followed by somewhat lower levels at 12 h and 24 h with an increase again at 96 h (Fig. 3). Gills obtained from these experiments were not perfused prior to dissection and have therefore not been analyzed for CA expression. However, perfused gills were

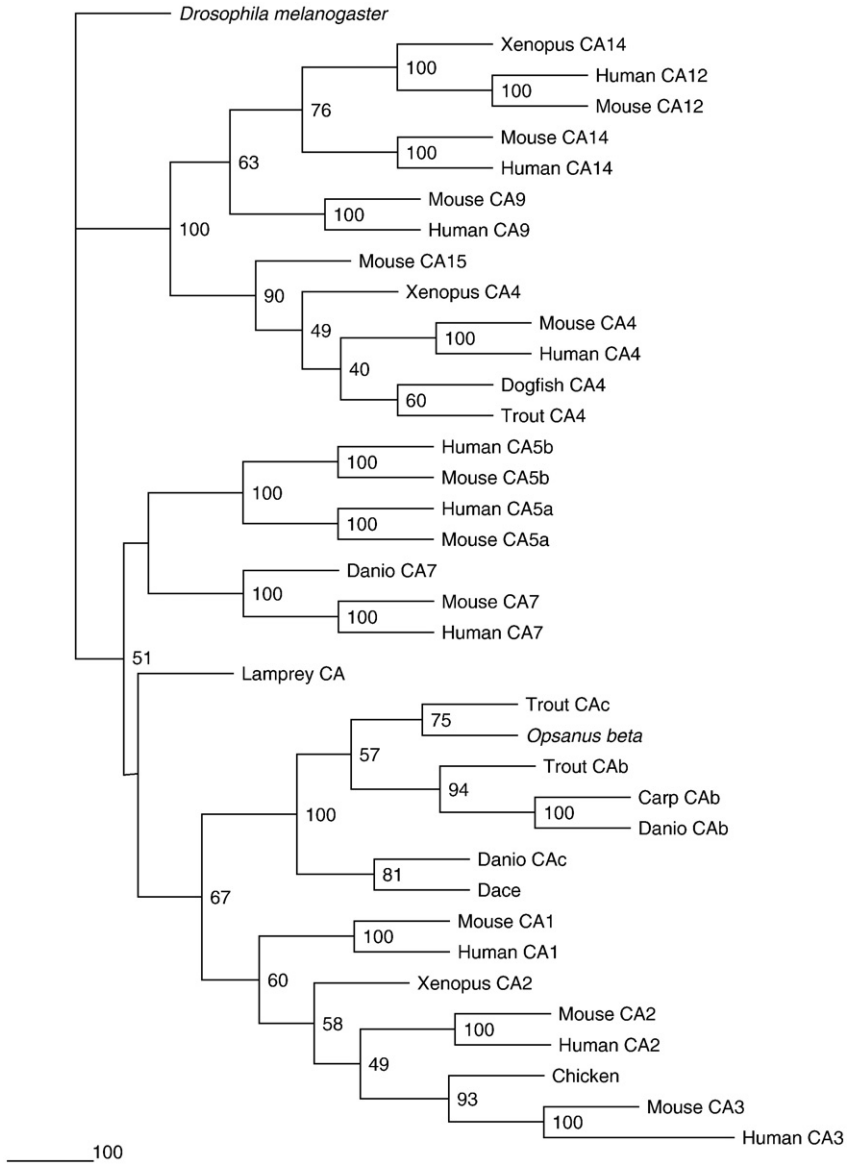

Fig. 1. A phylogenetic analysis of the cytoplasmic and membrane-bound CA isozymes of the α-CA gene family. The phylogenetic tree was constructed using neighbor joining with support for nodes assessed using bootstrap analysis (1000 replicates), and ordered using *Drosophila* CA as an outgroup. Bootstrap values below 50 were not included, denoting poor branch support.

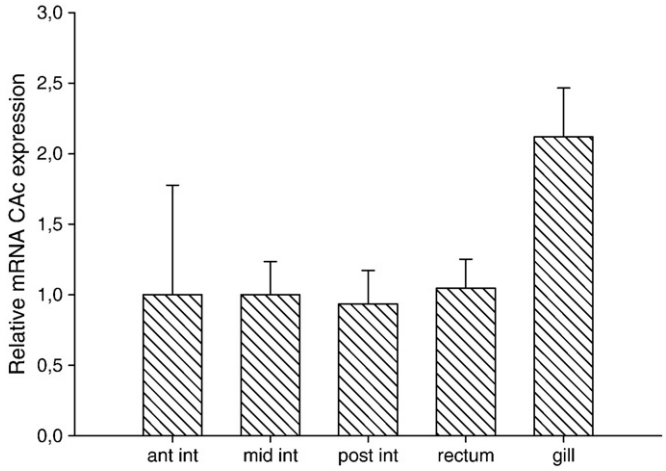

Fig. 2. Expression of cytoplasmic carbonic anhydrase mRNA relative to *EF1α* in gill, intestine and rectum of seawater acclimated gulf toadfish (*Opsanus beta*). Values are means ± SEM from $n = 6$ (for gill) and $n = 8$ (for other tissues) different fish.

obtained from fish sampled for CA enzyme activity analysis and showed no difference in expression between 40 ppt or 60 ppt after 14 days of exposure (Fig. 4). In contrast, CA enzyme activity in the cytosolic fraction of the gill tissue was significantly elevated in fish exposed to 60 ppt as was the case for all intestinal segments and the rectum (Fig. 5). Cytosolic CA activity is of the same magnitude as total CA activity illustrating that this isoform dominates the total CA activity of the gill and all intestinal segments (Fig. 5). Furthermore, it appears that the response to hypersalinity is less pronounced for total CA activity than for cytosolic activity (Fig. 5).

4. Discussion

We know from previous studies that the mammalian CAI, II and III evolved via a series of gene duplication events (Hewett-Emmett and Tashian, 1991) and that teleost fish cytoplasmatic CA duplicated in two isoforms: CAb and CAc, where CAb is expressed mainly in blood (Esbaugh et al., 2004; 2005; Lin et al., 2008) and CAc is expressed preferentially in gills (Esbaugh et al., 2005; Lin et al., 2008). The phylogenetic tree obtained suggests the cloned toadfish CA gene is the

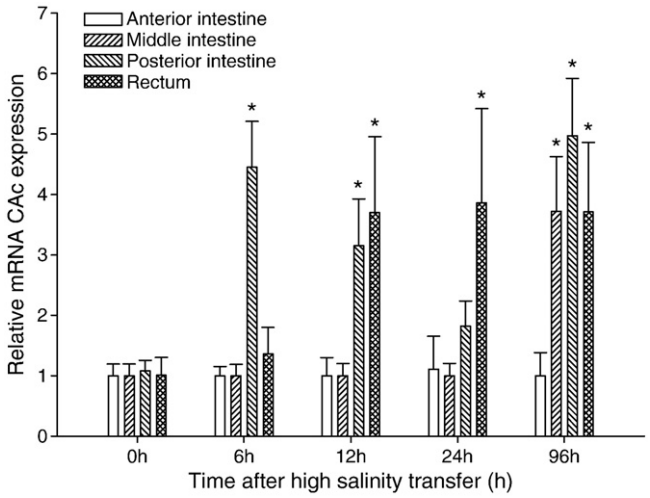

Fig. 3. Expression of gulf toadfish (*Opsanus beta*) cytoplasmic carbonic anhydrase mRNA relative to *EF1α* in anterior, middle, posterior intestine and rectum following transfer from seawater (40 ppt) to hypersaline water (60 ppt). Values are means ± SEM from $n = 8$ different fish. Statistically significant differences between control (0 h) and hypersalinity exposed animals are denoted by an asterisk (one-way ANOVA).

CAc isoform as it groups with fish CAc isoforms. The non-mammalian and mammalian cytoplasmic CA isozymes do not group together, in agreement with other trees reported for CA (Lund et al., 2002; Tufts et al., 2003; Esbaugh et al., 2004; 2005).

Commonly, studies of acclimation to salinity changes are performed on freshwater fishes acclimated to saltwater or vice versa. Studies examining the response of marine fish exposed to salinities exceeding normal seawater (~35 ppt) are less common. Recent studies have revealed that an intestinal Na^+:HCO_3^- cotransporter (tfNBC1) and possibly the Cl^-/HCO_3^- exchanger, tfSLC26a6 show elevated expression following such salinity challenges (Taylor and Grosell, 2009; Grosell et al., 2009).

Several studies show that CA is involved in osmoregulation in marine teleosts. Using pharmacological inhibitors previous studies (Dixon and Loretz, 1986; Wilson and Grosell, 2003; Grosell and Genz, 2006) demonstrated a key role of this enzyme for intestinal HCO_3^- secretion in marine teleost and euryhaline teleosts acclimated to seawater.

In this study we cloned and identified toadfish cytoplasmic CA (CAc) and the results obtained on activity and expression strongly suggest the involvement of this enzyme in hypersalinity responses of the intestinal tissue as well as gills of the gulf toadfish. In normal seawater gill CAc expression is two-fold higher than in intestinal or rectal tissue, showing similar expression. The observations of relatively high branchial CA expression are in agreement with earlier studies (Sender et al., 1999; Henry and Swenson, 2000; Esbaugh and Tufts, 2004; Esbaugh et al., 2005). However, salinity increase appears to affect expression considerably more in the intestine and rectum than in gill. In particular, the posterior intestine and rectum show pronounced expression increases following the exposure to hypersalinity indicating that elevated intestinal, more so than branchial CAc is important for survival in salinities above normal seawater.

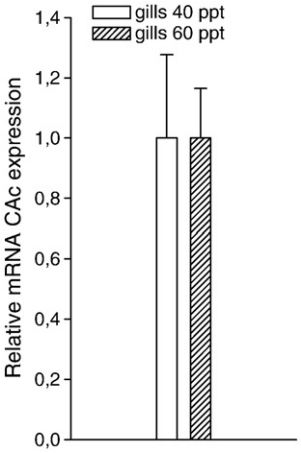

Fig. 4. Expression of gulf toadfish (*Opsanus beta*) cytoplasmic carbonic anhydrase m RNA relative to *EF1α* in gill following transfer from normal seawater (40 ppt) to hypersaline water (60 ppt). Values are means ± SEM from $n = 8$ different fish.

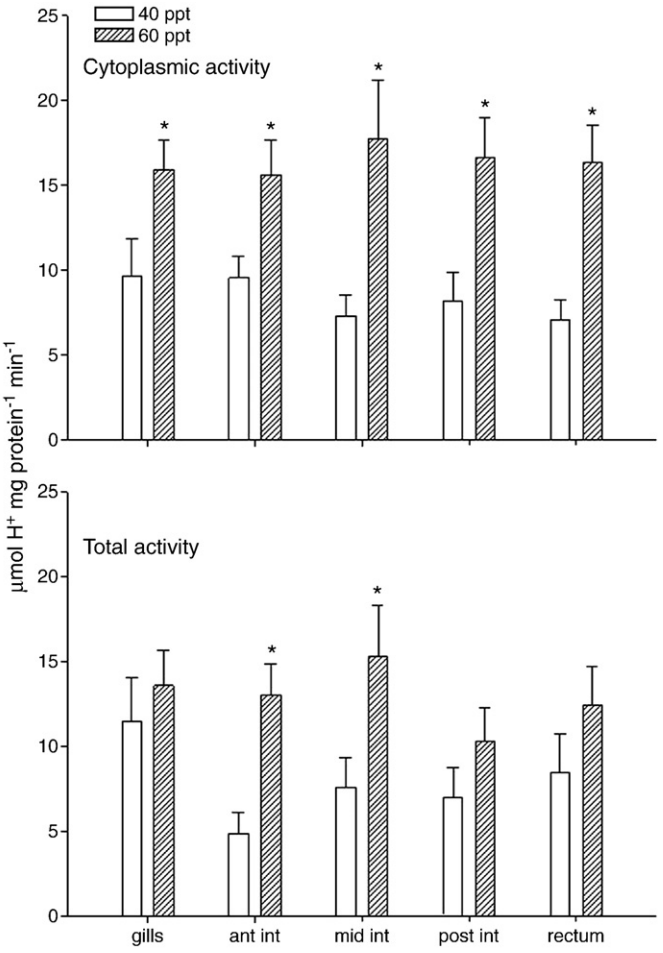

Fig. 5. Activity of cytoplasmic (upper panel) and total (lower panel) carbonic anhydrase in gulf toadfish (*Opsanus beta*) anterior, middle and posterior intestine, rectum and gills. Controls (40 ppt) and two weeks hypersalinity (60 ppt) exposed specimens. Values are means ± SEM from $n = 10$ different fish. Statistically significant differences between control and exposure to 60 ppt are denoted by an asterisk (two-way ANOVA).

Cytosolic carbonic anhydrase activity is comparable among tissues in seawater acclimated fish and also among tissues from fish held for two weeks at 60 ppt. However, in all tissues a significant increase in activity is observed after exposure to 60 ppt.

Comparing early mRNA expression and activity after two weeks of acclimation reveals a very different response to hypersalinity among tissues. The early mRNA expression increases substantially in the posterior intestine and rectum during 96 h post transfer with no apparent effect in the, anterior and middle intestine. In contrast, the enzyme activity increases to roughly the same extent in all segments of the intestine. Early expression in the gill was not assessed in the present study but no increase in branchial mRNA expression was observed after two weeks of acclimation although cytosolic enzymatic activity was significantly elevated at this time. In the case of the gill, since gill mRNA expression was only examined 14 days post transfer to 60 ppt, early transient expression changes might have occurred. In

the case of the intestine, our observations caution against conclusions based solely on early changes in mRNA expression and likely illustrates different rates of RNA and protein turnover in different intestinal segments during acclimation to elevated ambient salinity.

Our observations reveal that more distal segments of the intestine show the greatest mRNA expression response to elevated salinity with the highest expression recorded in posterior intestine and rectum. In contrast, the anterior intestine, which under normal seawater conditions is responsible for the bulk of intestinal HCO_3^- secretion, does not show significant shifts in CA expression, while the mid intestine increases only after 96 h. The present observations of distally elevated mRNA expression and activity of CAc is in contrast to observations of elevated NBC1 mainly in the mid intestine of toadfish exposed to 60 ppt (Taylor and Grosell, 2009) and both observations seems to disagree with the observation that most HCO_3^- is secreted by the anterior intestine *in vivo* under normal seawater conditions. Furthermore, Grosell and co-workers (2007) found that for rainbow trout activity in the anterior region was higher than in the posterior region of intestine. However, these discrepancies likely illustrate that the mid intestine and more distal segments are recruited for osmoregulatory purposes during exposure to salinities above those of normal seawater. Furthermore, NBC1 which is a component of transepithelial HCO_3^- transport and CAc which is central to HCO_3^- secretion from hydration of endogenous CO_2 are affected differently among intestinal segments during challenges by high salinity. This point to different mechanisms of HCO_3^- secretion in different intestinal segments. It appears that transepithelial HCO_3^- secretion may be more prevalent in the mid intestine and that CO_2 hydration is more important in the posterior intestinal and rectal segments of the intestinal tract, at least during exposure to hypersalinity.

In gills of seawater acclimated toadfish, CA activity levels were similar to all intestinal segments, which is in disagreement with results reported by Kültz and co-workers (1992) where the gill CA activity is substantially higher than intestinal CA activity. The present observations suggest that branchial CA may also play a role in osmoregulation in hypersaline environments. Genz et al. (2008) demonstrated that there is an increase in branchial net acid excretion in elevated salinity and that the increased acid extrusion at the gill compensates for increased transport of H^+ into the extracellular fluids occurring in response to intestinal processes associated with high salinity. The elevated branchial cytosolic CA activity confers an increased capacity for CO_2 hydration. Increased branchial capacity for CO_2 hydration in fish exposed to elevated salinity may increase the availability of HCO_3^- and protons and thus confer a greater ability to retain HCO_3^- by transport across the basolateral membrane and/or proton secretion across the apical membrane.

5. Conclusions

The CA reported herein is the CAc isoform, as confirmed by the phylogenetic analyses and is central for osmoregulation under hypersaline conditions. We demonstrate that early mRNA expression and later enzymatic activity are not uniformly correlated during exposure to hypersalinity. Our findings corroborate earlier observations of elevated branchial acid extrusion by fish in hypersaline water and implicate CO_2 hydration also in distal intestinal segments as important for survival under such conditions. Our observations suggest that the capacity for acid extrusion by Na^+/H^+ exchange or/and H^+-pumps must be elevated especially in the distal segments of the intestinal tract during exposure to hypersalinity to match increased hydration of CO_2 for osmoregulatory purposes.

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