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CARBONIC ANHYDRASE AND GLUTATHIONE PEROXIDASE. MOLECULAR PHYLOGENESIS AND PHYSIOLOGICAL IMPORTANCE FOR ENVIRONMENTAL STRESS RESISTANCE

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Riassunto esteso

Introduzione

I pesci vivono negli habitat più diversi: acque dolci (fino a 0,01 ppt), salate (fino a 100 ppt), stagnanti; in zone al di sopra dei 5.200 metri (Kottelat and Chu 1988) o nelle profondità abissali di 7000 metri (Nelson 1994). Alcuni vivono in zone soggette a forti maree e tollerano brevi escursioni sulla terraferma (Sayer 2005), altri resistono a temperature di circa 44°C (es. *Tilapia* in Africa) o di circa -2°C (es. *Trematomus* nelle acque antartiche) (Nelson 1994). Per vivere e riprodursi in ambienti tanto diversi hanno sviluppato una serie di adattamenti fisiologici, morfologici e comportamentali.

Il mio progetto di dottorato si è concentrato su due linee di ricerca: la filogenesi molecolare di due enzimi, la Carbonico anidrasi (CA, EC 4.2.1.1) e la Glutatione perossidasi (GPX, EC 1.11.1.9 e 1.11.1.12) di specie antartiche e intertidali; il coinvolgimento della CA nell'adattamento a condizioni di ipersalinità in una specie di teleosteo della zona intertidale.

I pesci antartici vivono in acque molto fredde, vicine al punto di congelamento (-1,8 °C), e con una concentrazione salina molto bassa (34,8 ppt) (Nelson 1994). Questi parametri si collocano all'estremo inferiore della scala di temperature e salinità delle acque dove vivono pesci di mare aperto la temperatura nelle acque temperate è di circa 20°C e la salinità è intorno a 40 ppt (Nelson 1994)- ma sono molto stabili anche nel periodo annuale. La zona di mare dove vivono questi organismi rappresenta una sorta di microambiente con minime variazioni (Eastman 2005). Una conseguenza diretta dei bassi valori di temperatura e salinità è una concentrazione di ossigeno molto più elevata nel caso delle acque antartiche rispetto a quelle temperate (valori nelle acque antartiche i valori vanno da un minimo di 159 a un massimo di 413 μmol/Kg (Meiner, Papadimitriou et al. 2009) mentre nelle acque temperate il minimo registrato e <20 μmol/Kg (Fuenzalida, Schneider et al. 2009)). La zona di acqua costiera dove vivono questi organismi, è circondata dalla corrente circumpolare antartica, formatasi circa 22-25 milioni di anni fa in seguito a una serie di movimenti tettonici e oceanografici, che crea una naturale barriera attraverso la quale i pesci non possono passare.(Eastman 2005) Si è formato cosi un unico sito dove si sono create delle nuove nicchie ecologiche

occupate da gruppi di pesci (i Nototenoidei) che si sono sviluppati *in situ.*(Anderson 1999)

I pesci studiati sono *Trematomus bernacchii, T. eulepidotus, T. lepidorinus* e *Cygnodraco mawsoni*, appartenenti alla famiglia dei notetioneidei, la fauna maggiormente rappresentata in termini di diversità, abbondanza e biomassa nell'Oceano Antartico (Eastman 1993). I membri di questa famiglia sono caratterizzati da diversi adattamenti fisiologici (ad es. le proteine antigelo) (Chen et al., 1997; Cheng C-HC, 2002)) e da un alto grado di diversità morfologica (Kock 1992; Eastman 2000) che li rendono adatti a sopravvivere in queste acque. I Nototenioidei sono endemici in Antartide ed è stato ipotizzato che rappresentino una radiazione adattativa avvenuta nell'Oceano Antartico (Briggs 1974; Briggs 1996) Esistono 8 famiglie di Nototenioidei per un totale di 44 generi e 129 specie. Di queste, 101 sono prettamente antartiche e solo 28 sono non antartiche (Bargelloni et al., 2000; Stankovic et al., 2002; Near et al., 2004).

La zona di mangrovia è situata tra mare e terra nella zona intertidale. La collocazione geografica di questo biotopo implica elevate temperature e di conseguenza la concentrazione di ossigeno disciolto e la salinità fluttuano ampiamente durante il corso della giornata per effetto combinato delle maree e della evaporazione dell'acqua (Lowe-McConell 1987; Morton 1989; Blaber 1997). Elevata salinità, elevate temperature e l'istaurarsi di condizioni di anaerobiosi nell'acqua sono le caratteristiche peculiari di questa zona (Kathiresan 2001). La maggior parte di pesci intertidali di questi areali, mostrano diversi adattamenti di tipo fisiologico, morfologico e comportamentale (Lewis 1970; Bridges 1993; Gibson 1996).

I *mudskippers* sono un gruppo di teleostei (Perciformes, Gobiidae, Oxudercinae) che vive tra le mangrovie; sono eurialini e adattati a cambiamenti estremi di salinità (Chew and Ip 1990), esposizione all'aria (Kok, Lim et al. 1998) ipossia (Chew SF 1990), e ad alte concentrazioni di ammonio (Ip, Randall et al. 2004). I *mudskippers* appartengono al gruppo monofiletico dei gobioidei (Winterbottom 1993; Thacker 2009) che ha subito una radiazione nel passaggio all'habitat marino.

L'*Opsanus beta* (Gulf toadfish) è un teleosteo marino distribuito lungo le coste tra il Golfo del Messico e il Sud America. Vive in acque stagnanti dove vi è un continuo mescolamento di acqua salata e acqua dolce. La continua evaporazione crea un ambiente con salinità fluttuante (Lirman and Cropper 2003); ad esempio le medie di salinità

calcolate nella zona di Florida Bay vanno da un minimo di 24.2 ppt in novembre a un massimo di 41.8 ppt in luglio (Kelble, Johns et al. 2007). Il toadfish tollera salinità che vanno da 5 a 60 ppt in condizioni di laboratorio (McDonald and Grosell 2006). All'aumentare della salinità si osserva un aumento della richiesta di assorbimento di sale dall'intestino; questo ha un significativo impatto sul bilancio acido-base dovuto al cambiamento nell'escrezione di CO_2 nell'intestino (Genz, Taylor et al. 2008).

La Glutatione Perossidasi

La Glutatione perossidasi GPX) è una famiglia di isozimi che catalizzano la riduzione degli idroperrosidi organici ad acqua, o alla corrispondente sostanza alcolica, usando il glutatione ridotto (GSH) come donatore di elettroni. Di questo enzima, che può essere seleno-dipendente o indipendente, ne sono state caratterizzate 4 diverse isoforme: GPX1 localizzata in fegato, polmoni e reni, GPX2 gastrointestinale, GPX3 trovata in reni, polmoni, epididimo, vasi deferenti, placenta, vescicole seminali,cuore e muscolo e GPX4 (fosfolipidica) distribuita largamente nei tessuti (Hochachka and Lutz, 2001; Margis et al., 2008). L'importanza della GPX è di preservare le cellule dai possibili danni dovuti alla produzione di H_2O_2 prodotto nei mitocondri come conseguenza della fosforilazione ossidativa. In diversi siti lungo la catena respiratoria mitocondriale, l'ossigeno subisce una riduzione parziale, generando l'anione superossido che è il primo di una serie di radicali (O_2^-), il radicale idrossilico (OH·), l'ossigeno singoletto ($^1O_2$) e il perossido di idrogeno (H_2O_2)) (Miller 1993). I radicali prodotti (ROS, reactive oxigen species) interagendo con diversi target intracellulari (lipidi, proteine e acidi nucleici) attivano meccanismi di morte cellulare e inducono varie disfunzioni cellulari ritenute responsabili di molteplici patologie. In condizioni di ipossia o iperossia la produzione di ROS è maggiore (Fink and Scandalios 2002).

La Carbonico Anidrasi

La carbonico anidrasi riveste un ruolo fondamentale nella reazione reversibile di idratazione, deidratazione della CO_2 con produzione di H^+ e HCO^{3-} ($CO^2+H_2O \rightarrow H^+ + HCO^{3-}$). La funzione principale della CA è la regolazione acido-base e

l'osmoregolazione a livello intestinale e branchiale (Geers and Gros, 2000; Esbaugh and Tufts, 2006). La CA dei pesci studiati, probabilmente, ha sviluppato un particolare adattamento, legato all'osmoregolazione, dovuto agli ambienti ipo (oceano Antartico) e ipersalino (zona intertidale) in cui vivono.

Nei mammiferi si conoscono 16 differenti isozimi che differiscono per proprietà cinetiche, distribuzione tissutale e localizzazione subcellulare. Le diverse isoforme della famiglia delle α-CA sono la I, II, III, V, VII and XIII citoplasmatiche, la IV, IX, XII,XIV, XV legate in membrana (Esbaugh and Tufts 2006). La CAII sembra essere in uno stato ancestrale nei pesci, mostrando alta attività, fino ai teleostei dove apparentemente si è duplicata in due diverse isoforme, la CAb (eritrocitaria) e la CAc (citoplasmatica) (Esbaugh et al., 2004; Esbaugh et al., 2005).

Il lavoro svolto durante il mio dottorato può essere suddiviso in tre parti principali:

1. Il clonaggio e il sequenziamento di GPX di pesci antartici per individuare mutazioni aminoacidiche nella sequenza della proteina caratteristiche di queste specie e fare una ricostruzione filogenetica dell'enzima. Anche in questo caso si è partiti con un'estrazione di RNA da branchie. La ricostruzione filogenetica ci ha permesso di indagare la storia evolutiva di tali proteine confrontandole con sequenze di GPX di altri teleostei e di altri vertebrati e prendendo in considerazione la storia evolutiva degli organismi. I pesci sono stati campionati durante la XIV (1998-1999), la XVII (2001-2002) e la XXI (2005-2006) campagna italiana in Antartide.

2. Il clonaggio e il sequenziamento di CA di pesci antartici e di zone intertidali (mangrovieti e acque ipersaline). Questo è stato ottenuto partendo dall'estrazione di RNA da tessuto branchiale o intestinale di tali pesci. Ottenute le sequenze delle CA, queste sono state utilizzate per un'analisi filogenetica. Le sequenze sono state confrontate tra loro e con le sequenze di CA disponibili in rete per evidenziare la presenza di zone conservate o meno ed è stata fatta una ricostruzione tridimensionale per vedere e confrontare la distribuzione di potenziale elettrico delle stesse. La ricostruzione filogenetica è servita per delineare un'ipotetica evoluzione di tali enzimi in confronto con gli enzimi di altri teleostei e di altri vertebrati tenendo conto della storia evolutiva di tali organismi. (I pesci sono stati campionati durante la XIV (1998-1999), la XVII (2001-2002)e la XXI (2005-2006) campagna italiana in Antartide.)

3. Il clonaggio e il sequenziamento della CA del Gulf Toadfish (Opsanus beta), un teleosteo che vive in zona intertidale con tendenza all'ipersalinità. Una volta ottenuta la sequenza (partendo anche in questo caso da estrazione di RNA da tessuti) è stata fatta una ricostruzione filogenetica e delle analisi di attività ed espressione dell'enzima. I tessuti analizzati sono intestino (anteriore, medio e posteriore), retto e branchie. L'analisi è stata fatta su campioni controllo (stabulati alla stessa salinità dell'acqua in cui vivono (40ppt) e su pesci stabulati in condizioni d'ipersalinità (60ppt) per 6-12-24-48-96 ore (per quanto riguarda le misure di espressione nei vari segmenti di intestino e retto) o due settimane (per quanto riguarda misure di espressione e attività su branchie e di attività sui segmenti di intestino e retto). Questo studio è stato svolto per indagare il coinvolgimento della CA a livello intestinale e branchiale nell'osmoregolazione di pesci d'acqua salata se sottoposti a un ambiente ipersalino. Per questa parte del lavoro mi sono recata da aprile a ottobre 2009 presso il laboratorio del professor Martin Grosell (RSMAS, University of Miami, Florida, USA).

Risultati e Discussione

Glutatione Perossidasi

La sequenza della GPX1 selenio-dipendente è stata ottenuta per i pesci antartici *Trematomus bernacchii* (946 bp), *T. lepidorhinus* (946 bp), *T. eulepidotus* (945 bp) e *Cygnodraco mawsoni* (950 bp). Tutte le sequenze sono caratterizzate da un *open reading frame* (ORF) di 191 amminoacidi. In posizione nucleotidica 174-176 è presente la tripletta TGA che codifica per la selenocisteina, un amminoacido facente parte della triade catalitica.

La proteina sequenziata corrisponde all'isoforma 1 (citoplasmatica) perché dalla ricostruzione filogenetica ottenuta con il metodo del NJ le sequenze si pongono tutte in un *cluster* vicino all'isoforma 1 e separato dal gruppo dalle GPX2.

Si può ipotizzare un'origine monofiletica all'interno dei teleostei antartici, che sono un clade completamente separato. Questo enzima sembra essersi evoluto nei pesci antartici indipendentemente dalle altre specie di teleostei.

La ricostruzione filogenetica ottenuta conferma le odierne analisi filogenetiche sull'evoluzione delle GPX (Margis et al., 2008; Toppo et al., 2008). Le varie isoforme risultano separate da valori di *bootstrap* che si approssimano al 100%, dimostrando che le

isoforme 1, 2 e 3 sono più vicine filogeneticamente fra loro, mentre le isoforme 7 e 8 sono più affini alla GPX4.

Il *cluster* dei teleostei antartici risulta indipendente e ben separato dagli altri con valori di *bootstrap* del 100%, avvalorando l'idea di un'origine monofiletica della proteina in questo gruppo.

All'interno del ramo dei teleostei antartici, le distanze evolutive appaiono molto brevi, indicando che la diversificazione della GPX nelle diverse specie è avvenuta in tempi piuttosto recenti. In questo gruppo le relazioni evolutive non sono completamente risolte, nonostante alcuni nodi siano sostenuti da alti valori di *bootstrap*.

La GPX di *C. mawsoni* risulta essere il *sister group* delle GPX delle specie appartenenti al genere *Trematomus*. Analisi filogenetiche basate sullo studio delle subunità 16S e 12S dell'rRNA mitocondriale, indicano *Cygnodraco mawsoni* come *sister group* della famiglia dei Nototheniidae, alla quale appartiene anche il genere *Trematomus* (Bargelloni, Marcato et al. 2000), confermando la ricostruzione filogenetica.

Il lavoro di (Epp, Ladenstein et al. 1983) ha determinato per la prima volta la struttura cristallografica della GPX1 bovina e ha evidenziato la triade catalitica rappresentata da selenocisteina (40), glutammina (75) e triptofano (153). E' stato proposto che nel sito attivo la glutammina e il triptofano siano legati mediante ponti idrogeno alla selenocisteina e che attivino l'elemento redox (il selenolo) proprio grazie a questo legame che dovrebbe facilitare l'attacco nucleofilico dell'idroperossido. Questi aminoacidi sono, infatti, altamente conservati anche nei teleostei. I residui coinvolti nella formazione dei foglietti β e delle α-eliche rimangono, tranne alcune eccezioni, invariati. La regione che sembra avere un ruolo fondamentale nella capacità di dimerizzazione della proteina è rappresentata dai residui 89-95, che mancano nella GPX4, che per questo motivo presenta una struttura monomerica (Scheerer, Borchert et al. 2007). Questi residui sono conservati anche nei teleostei, anche se con alcune eccezioni. La Val90 è stata sostituita nei soli teleostei antartici, da un altro amminoacido alifatico, la leucina. Mentre negli anfibi e nei mammiferi in posizione 94 è presente una glicina, nei teleostei troviamo quattro amminoacidi diversi, tutti residui polari: nei teleostei antartici troviamo una lisina (carica positiva), in *H. molitrix* un aspartato (carica negativa), in *D. rerio* un glutammato (carica negativa) e negli altri teleostei una asparagina (senza carica).

Possiamo ipotizzare che tali cambiamenti aminoacidici abbiano un effetto sulla velocità e sull'efficienza della reazione catalizzata dalla GPX.

Carbonico anidrasi

La carbonico anidrasi di tre specie antartiche e di due specie di zona intertidale è stata sequenziata completamente (*Trematomus bernacchii* (1062 bp)*, T. lepidorinhus* (1656 bp)*, T. eulepidotus* (1563 bp) per le specie antartiche e *Periophtalmus sobrinus* (1217 bp) e *Opsanus beta* (1827 bp) per le specie intertidali). Una quarta sequenza, mancante di una parte in 3', è stata ottenuta per *Cygnodraco mawsoni* (713 bp), (specie Antartica) tutte con un ORF di 260 aminoacidi (GenBank accession number: GQ443602 (*T. bernacchii*); GQ443601 (*T. lepidorinhus*); GQ443600 (*T. eulepidotus*); GQ443603 (*Periophtalmus sobrinus*). Queste sequenze saranno disponibili a partire da settembre 2010 mentre per *Opsanus beta* (GQ443599) la sequenza è già disponibile in rete)

Le sequenze mostrano un'identità piuttosto alta con le sequenze di CAII disponibili in database (dall'80% al 72%). Per questo e perché nell'albero ottenuto con il Neighbor Joining (NJ) raggruppano nel cluster delle CAII, possiamo ritenere che si tratti dell'isoforma II. Nell'albero ottenuto (Fig. 2) sono ben visibili le separazioni tra il gruppo delle CA di membrana e quelle citoplasmatiche. Tra quest'ultime vi è un'altra separazione, tra le CAII citoplasmatiche (CAIIc) e le CAII citoplasmatiche eritrocitarie (CAIIb). Possiamo ipotizzare che la CA di *Opsanus beta* sia l'isoforma CAIIc, ovvero che si tratti dell'isoforma citoplasmatica e non di quella eritrocitaria (CAIIb), perché nella ricostruzione filogenetica è raggruppata insieme alla CAc di trota. La CA del mudskipper, invece, raggruppa con le isoforme CAb di trota, carpa e zebrafish. Un'altra divisione ben delineata e mostrata anche nell'albero ottenuto da Esbaugh et al., 2006, è quella esistente tra mammiferi e non mammiferi.

Le CA di pesci antartici raggruppano insieme in un *cluster* separato dagli altri e ben supportato da alti valori di bootstrap, sottolineandone la stretta relazione filogenetica. Anche l'icefish (*Chionodraco hamatus*) raggruppa in questo *cluster*.

Gli aminoacidi che compongo la triade catalitica (His 94, 96, 119) e quelli che mantengono la struttura funzionale della proteina (Thr 199 e Glu 106) sono conservati, mentre possiamo notare dei cambiamenti a livello aminoacidico propri delle sequenze di carbonico anidrasi dei pesci Antartici. In posizione 10-11 si trovano una Ala e una Asn

conservate solo nelle sequenze di CA di pesci antartici, come in posizione 157 dove vi è una Ser e in posizione 190-191 con una Gly e una Cys.

Carbonico anidrasi in Opsanus beta

La carbonico anidrasi sequenziata (GenBank accession number GQ443599) è di 1827 paia di basi con un ORF di 260 aminoacidi. La sequenza mostra un'identità del 78% con *Oncorhynchus mykiss* e *Pseudopleuronectes americanus*, del 77-76% con le altre sequenze di CA di teleostei, e del 63-59% con anfibi e mammiferi. Le analisi filogenetiche effettuate con il metodo del NJ raggruppano la CA con le isoforme citoplasmatiche degli altri teleostei e la separano dalle isoforme di membrana (CA IV, IX, XII, XIV, XV) e dalle sequenze di mammiferi e di altri tetrapodi, in accordo con quanto riportato da altri studi. La CAII può essere suddivisa in due isoforme, la CAIIc (citoplasmatica) e la CAIIb (citoplasmatica eritrocitaria). La ricostruzione filogenetica ottenuta suggerisce che l'isoforma sequenziata sia di tipo CAIIc poiché raggruppa con le CAIIc di altri pesci.

Su branchie, intestino (anteriore, medio e posteriore) e retto sono state condotte misure di espressione dell'mRNA tramite qPCR. Intestino e retto mostrano simile espressione in pesci acclimatati all'acqua salata mentre le branchie mostrano un'espressione più elevata. Nei pesci sottoposti a ipersalinità (60 ppt), si osservano alti livelli di espressione rispetto al controllo (40 ppt) nell'intestino medio e posteriore e nel retto. In particolare l'intestino medio risponde per ultimo all'ipersalinità, dopo 96 ore di esposizione, mentre il retto mostra una maggiore attività già dopo 12 ore di esposizione mantenendosi stabile per tutto il tempo. L'intestino posteriore mostra un rapido incremento di espressione dopo 6 ore di esposizione con un decremento dopo 12 e 24 ore seguito da un altro incremento dopo 96 ore.

Per quanto riguarda le branchie, è stato possibile fare misure di espressione su branchie perfuse solo dopo due settimane di stabulazione all'ipersalinità. I dati ottenuti non mostrano un incremento nell'espressione in pesci a 40 ppt o a 60 ppt.

L'attività, misurata su tessuti di pesci controllo (40 ppt) e su tessuti di pesci esposti per due settimane a 60 ppt mostra un significativo incremento nella frazione citosolica nei tessuti dopo l'esposizione. L'attività totale, confrontata con la citosolica, mostra un minor incremento.

Diversi studi mostrano che la CA è coinvolta nell'osmoregolazione in teleostei marini. Usando inibitori (Grosell and Genz 2006), è stato dimostrato che la CA ha un ruolo chiave per la secrezione intestinale di HCO_3^- in teleostei marini.

In questo studio è stato dimostrato il coinvolgimento di questo enzima in tessuti intestinali e branchie in risposta all'ipersalinità. Il confronto tra l'espressione e l'attività registrata rivela una diversa risposta. L'espressione di mRNA incrementa nell'intestino posteriore e nel retto con effetto non evidente a livello branchiale, intestino anteriore e medio. Invece, l'attività dell'enzima aumenta visibilmente in tutti i tessuti. Le nostre osservazioni rivelano, quindi, una maggiore espressione nei tessuti distali dell'intestino. Invece, la parte anteriore dell'intestino che sotto normali condizioni di salinità è responsabile della maggior parte dell'escrezione di HCO_3^- non mostra un visibile cambiamento di espressione nei tessuti di pesci acclimatati a ipersalinità. Questo è in contrasto con quanto trovato nella trota (Grosell, Gilmour et al. 2007), dove l'attività era maggiore nella regione anteriore dell'intestino piuttosto che nella posteriore.

Nelle branchie, l'attività della CA è confrontabile con quella registrata nelle diverse regioni dell'intestino.

Le nostre osservazioni possono suggerire un coinvolgimento della CA branchiale nell'osmoregolazione ad ambienti ipersalini. L'incremento della capacità della CA branchiale di idratare la CO_2 in pesci sottoposti a ambienti ipersalini, può aumentare la disponibilità di HCO_3^- e protoni e quindi conferire una maggiore abilità di trattenere HCO_3^- trasportata attraverso la membrana basolaterale e/o la secrezione di protoni attraverso la membrana apicale.

Contents

List of papers

The thesis is based on the following papers which will be referred to by their roman numbers:

I. Giovanna Sattin, Gianfranco Santovito, Arnaldo Cassini: **Molecular and phylogenetic analysis of Antarctic fish Glutathione Peroxidase**

II. Giovanna Sattin, Stefano Marino, Luigi Bubacco, Gianfranco Santovito, Mariano Beltramini: **Molecular phylogenetic analysis of Carbonic anhydrase in teleosts. Insights into molecular adaptations to temperatures**

III. Giovanna Sattin, Edward M. Mager, Mariano Beltramini, Martin Grosell: **Cytosolic carbonic anhydrase in the Gulf toadfish is important for tolerance to hypersalinity**

Abstract and aim of thesis

Glutathione peroxidase (GPX, EC 1.11.1.9 and EC 1.11.1.12) and Carbonic anhydrase (CA, EC 4.2.1.1) are two enzymes important for a variety of adaptation/tolerance processes of organisms. CA plays a key role in osmoregulation when GPX is one of the most important enzymes involved in protection of the organism against oxidative damage. In my thesis I studied GPX and CAs from Antarctic teleosts species of Notothenidae and Bathydraconidae (Paper I-II) and CAs from intertidal teleosts (*Periophtalmus sobrinus,* Gobidae*,* and *Opsanus beta,* Batrachoididae (Paper II-III). Firstly, I compared the obtained sequences with those available in database for teleosts and other vertebrates (human, mouse and bird) to reconstruct the molecular phylogeny of two enzyme in teleosts (Paper I-II). Finally, I have investigated the role of CA for the osmoregulation in seawater toadfish (*Opsanus beta*) exposed to hypersalinity (Paper III).

The GenBank database, few sequences of GPX and CAs of temperate teleosts are available and no data are available for Antarctic or intertidal fish were not present. In particular, I choose to study those species because they live in environments with peculiar chemical/physical parameters that influenced, during the evolution, their physiological, morphological and behavioural characteristics.

The Antarctic fish (Papers I-II) live in a well delimited geographic zone. The Southern Ocean is comprised from the Antarctic continent to the Polar Front, a curved current continuously encircling Antarctica where cold, northward-flowing Antarctic waters meet and mix with the relatively warmer sub-Antarctic waters. In this limited zone, macro evolutionary events happened, such as radiation of some fauna components. Most of the fish species living in this zone (like nototenioidae) are presents only there. Temperature (-1.9 °C) and salinity (34.8 ppt) are lower than in temperate Oceans (Legg, Briegleb et al. 2009). As a consequence, the oxygen concentration is higher (9 mg l^{-1} (Meiner, Papadimitriou et al. 2009)) in these waters because of the higher solubility of the gas. Furthermore, these parameters are constant during the year. In this perspective, is high the interest to study the evolution of some proteins such as of GPX (Paper I), linked to hyperoxia, and of CAs (Paper II), linked to osmoregulation,

to investigate if two enzymes have been an independent evolution and if them aminoacidic sequences are modified compared to sequences of temperate teleosts.

The intertidal species (mudskipper *Periophtalmus sobrinus* (Paper II) and toadfish *Opsanus beta* (Paper III) both live in mangrove zones. Temperature (the average 28.9°C in Kenya (Schmitz, Jansen et al. 2007) and in 25.8°C Florida (Kelble, Johns et al. 2007)) and salinity (the average 32.4 ppt in Kenya (Schmitz, Jansen et al. 2007) and 33.0 ppt in Florida (Kelble, Johns et al. 2007)) are higher with respect to temperate oceans and these parameters, in contrast to Antarctic habitat, are very fluctuating. Gazy Bay (Kenya), where mudskipper lives, is subject to seasonal fluctuation due to tides. Florida Bay (Florida), where toadfish lives, is subject to seasonal fluctuation due to wet and dry season. The mudskipper was sampled in Gazy Bay (during summer of 2007), and toadfish was sampled in Florida Bay (during summer of 2009).

My data show that both GPXs and CAs aminoacidic sequences are conserved in all teleosts (Paper I-II). Just a few aminoacidic changes were found in Antarctic teleosts aminoacidic sequences. Considering the active site and he aminoacidic zone toward the catalytic centre of GPX (Paper I), only the polar Arg^{182}, conserved in all teleosts GPX-1, changes in a hydrophobic Lys in Antarctic fish enzyme (Aumann, Bedorf et al. 1997).

In CA sequences the analysis has been focused on residues that are included within 10 Å of the zinc atom (Paper II). They are almost all conserved, with only a few aminoacidic substitutions but maintaining the same properties.

Consequently we assume that the features of active site and the aminoacidic sequence toward the catalytic centre of both enzymes are well conserved in teleosts.

The phylogenetic tree has different topology for GPX and CA sequences (Paper I-II). In the GPX the Antarctic teleosts group together, while in the CA tree they are divided in two different groups. The topology of GPX of Antarctic teleosts is the same as that of Antarctic teleosts obtained by molecular data (16S rRNA. (Near, Pesavento et al. 2004). This suggests that the CAs evolved in a separate way.

In the last paper (Paper III), I measured the expression and activity of CA linked to osmoregulation. It was not possible to perform the same analysis for GPXs and CAs of other fish sequenced because the samples used were frozen while to measure activity I needed fresh samples. I found a correlation between hypersalinity and increase of CA

activity and expression in gills and intestine. This suggests an involvement of gill and intestine CA in regulation of seawater fish exposed to hypersalinity.

Introduction

Some fish developed particular physiological and morphological adaptation. They may tolerate a wide range of temperature, salinity, dissolved oxygen concentration, etc. Many species have acquired air-breathing organ to survive in stagnant tropical swamps; others demand well oxygenated waters to sustain its life. Therefore they are object of different studies of behaviour, ecology, evolution, genetics and physiology (Nelson 1994).

Also molecular component of this organism have been subjected to evolution and are extensively studied.

Object of my study are two enzymes involved in protection from oxygen damage (Glutathione Peroxidase-GPX) (Paper I) and osmoregulation (Carbonic Anhydrase-CA) (Paper II-III) in fish living in Antarctic Ocean (Paper I-II) and in intertidal zone (Paper II-III). Fish subject of this study are: Antarctic teleosts living in Southern Ocean; mudskipper living in Kenya and toadfish living in Florida.

Antarctica

In the beginning of the early Miocene (25–22 million years ago) the Antarctic shelf was subjected to a series of tectonic and oceanographic events that altered geography and faunal composition. Antarctica gradually became isolated and cold and the expansion of ice shelf led to destruction and disturbance of inshore habitat (Anderson 1999). Changes of habitat and trophic structure of the Antarctic ecosystem led to the extinction of many fish present in Eocene. Thus the diversity of the fauna have been reduced and becoming Antarctica isolated, new ecologically niches became available to other fish that were diversifying (notothenioids) or immigrating (liparids and zoarcids), developing cold-water ecosystem.

Fish living today in Antarctic Ocean are exposed to the lowest temperatures (-1.9 °C) and lowest seawater salinity (34.8 ppt) (Legg, Briegleb et al. 2009). This environment is characterized by great stability of physical parameters as temperature, salinity (with no freshwater inflows and only very minor salinity changes during the

annual cycle of pack ice formation and melting) and oxygen content (which is uniformly high). The continent is geographically delimited by an oceanographic feature: the Antarctic Polar Front. In the area between the Polar Front and the continental margins we can recognize macro evolutionary events such as radiations of some fauna components. By this, the Antarctic waters might be considered a particular evolutionary habitat because there a concentration of diversity has been developed in a rather small number of species (Eastman 1993). It is not the number of species that distinguishes the Antarctic fish from all other in the world, but the nature of the fish biodiversity.

There are currently 322 recognized species of Antarctic fish confined to 19 families, a small number considering the global numbers. The notothenioids is the most speciose Taxa; they are the indigenous Southern Hemisphere component of fauna that evolved in Antarctica (Andriashev 1965). The notothenioids dominate the fauna in terms of diversity, abundance and biomass and dominate by a single taxonomic group is unique among shelf faunas of the world. Notothenioids were subject to radiation in the subzero waters of the continental shelf (Eastman, 1993; Clarke and Johnston, 1996; Eastman, 2000) and may form a special type of adaptive radiation (Eastman and McCune 1969; Eastman and Clarke 1998): a disproportionately high number of closely related species that have evolved rapidly within a circumscribed area where most species are endemic. The eight notothenioid families encompass a total of 44 genera and 129 species, 101 Antarctic and 28 non-Antarctic (Bargelloni et al., 2000; Stankovic et al., 2002; Near et al., 2004). The different species that live in those waters developed particular morphological and physiological adaptations. For examples the origin and acquisition of antifreeze glycopeptides (Chen et al., 1997; Cheng and DeVries, 2002) and the loss of erythrocytes and haemoglobin (Cocca et al., 1995; Zhao et al., 1998; Detrich, 2000; Di Prisco et al., 2002) and the variable patterns of myoglobin expression in muscle tissues (Sidell et al., 1997; Moylan and Sidell, 2000) of white blooded channichthyids.

Channichthyidae (icefishes) a clade that contains 16 recognized species is one of the most interesting notothenioid lineages (Iwami and Kock, 1990; Eastman, 2000; La Mesa et al., 2002). Most channichthyids species are confined to the Antarctic region, but at least three species are found outside of this region in the Kerguelen Islands and the Falkland Islands (Iwami and Kock 1990). Channichthyids are well known for being the only group of vertebrates that lacks oxygen-transporting haemoglobin in the blood. The

cardiovascular system of channichthyids is thought to have compensated for the lack of haemoglobin and the low oxygen-carrying capacity of the blood through adaptations that include a greater blood volume and higher cardiac output whit comparison to other notothenioids (Hemmingsen 1991).

The growing database of nucleotide sequences is contributing more characters for phylogenetic relationships of notothenioids (Chen et al., 1997; Bargelloni et al., 2000; Near et al., 2004). Phylogenetic analysis based on morphology and genome agrees that channichthyids are a monophyletic group, and are one of the most derived clades of notothenioids (Iwami and Kock, 1990; Balushkin, 2000; Bargelloni et al., 2000). Phylogenetic hypotheses inferred from morphology place the notothenioid family Bathydraconidae as the sister lineage of the Channichthyidae (Iwami and Kock 1990; Balushkin 2000). The most important result regarding the phylogenetic relationships of notothenioids is the demonstration of monophyly for both the Notothenioid and the Nototheniidae (Bargelloni, Ritchie et al. 1994). Phylogenetic relationships within Channichthyidae have been investigated using both discretely coded morphological characters (Balushkin 2000; Voskoboinikova 2000) and mtDNA sequence data (Chen et al., 1997; Near et al., 2003). Sequence data indicate bathydraconids as closely related to channichthyids (Bargelloni and Lecointre 1998; Bargelloni, Marcato et al. 2000).

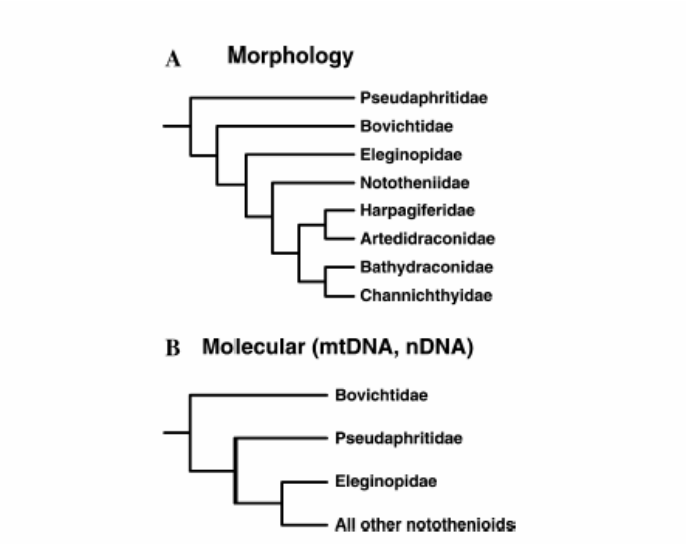


Figure 1: (A) Relationships of notothenioid families resulting from analyses of morphological characters (Balushkin 1992; Balushkin 2000). (B) Consensus of relationships among basal notothenioid lineages resulting from analyses of mtDNA and nuclear gene sequences (Bargelloni, Marcato et al. 2000; Cheng and DeVries 2002). (Figure from (Near, Pesavento et al. 2004)).

Intertidal zone

A wide variety of fish species has successfully colonised intertidal shores and adapted to the fluctuating environmental conditions that are characteristic of intertidal habitats (Gibson 1996). The mangrove areas are located at the interface between land and sea and thus are influenced by both terrestrial and marine factors. They grow in tropical and sub-tropical latitudes where they live in conditions of high salinity, extreme tides, strong winds, high temperatures and muddy anaerobic soils. Living in this environment they are well adapted to deal with natural stressors (e.g., temperature, salinity, anoxia, UV).

Mangrove forests are very unstable habitats in which temperature, salinity and dissolved oxygen fluctuate greatly (Lowe-McConell 1987; Morton 1989; Blaber 1997). In this habitat, there is also the risk of predation by both terrestrial and aquatic predators. Many intertidal fish, that are able to withstand these environmental changes, show morphological, physiological and behavioural adaptations which enable them to survive and reproduce in a habitat subject to regular change (Lewis, 1970; Bridges, 1993; Gibson, 1996; Ip et al., 2004).

Fish living in the mangrove must adjust their physiology to temporal and spatial variability of physical and chemical properties of this environment, and some species possess specific adaptations to deal with this. (Taylor, Davis et al. 1995). Fish without such specific adaptations may respond behaviourally to physical cues that indicate physically or chemically stressful microhabitats. This can lead to distinct distributional patterns (Blaber 1997). For example, (Heath, Turner et al. 1993) demonstrate experimentally that thermal cues affect fish distributions within mangrove ponds. Hypoxia, which affects plasma osmolality, plasma chloride ion concentration, and in fish hematocrit (Peterson, Kwun et al. 1990; Peterson and Gilmore 1991) can also influence their distributions.

In the environment where they live, intertidal fish experience episodic or chronic oxygen stress. For example pressures of aerial roots drop during high tide, probably due to removal of CO_2 from gas spaces during flooding. As the waters recede on the low tide, a rapid influx of air may take place. Moreover, high methane levels in the

sediments, can be associated with anoxia in fish living in mangrove environments (Sotomayor, Corredor et al. 1994).

In order, in mangrove with strong riverine input, the combined effects of evaporation and transpiration may remove much of the fresh water entering the system at high salinity at the end of dry period (Kathiresan 2001).

The representative species living in mangrove habitats that I studied are the *Gobidae Periophtalmus sobrinus* and the *Batrachonidae Opsanus beta.*

The suborder Gobioidei comprises a significant fraction of perciform diversity. The total number of gobioid species range from 1590 (Eschmeyer 2008) to 2211 (Nelson 2006). Gobioidei is traditionally divided into nine families: Rhyacichthyidae, Odontobutidae, Xenisthmidae, Eleotridae, Gobiidae, Microdesmidae, Ptereleotridae, Kraemeriidae, and Schindleriidae (Miller, 1973; Springer, 1983; Hoese, 1984); (Hoese and Gilla, 1993; Johnson and Brothers, 1993; Thacker and Schaefer, 2009). Gobiidae includes Gobiinae, Gobionellinae, Sicydiinae, Oxudercinae (mudskippers: Periophthalmus), and Amblyopinae (Hoese 1984). Abundant morphological and molecular characters indicate that Gobioidei is a monophyletic group (Winterbottom 1993). Here is original and high diversity of Gobiidae is due to a shift from freshwater to marine habitats early in the clades history. Such a radiation would be expected to generate switch to marine habitats is still consistent with an overall increase in diversification rate through time, yielding the greater species diversity in Gobiidae (Thacker 2009).

The Batrachoididae (toadfish) are comprised of 19 genera and 69 species inhabiting temperate, sub-tropical and tropical environments worldwide (Nelson 1994). Besides the family is mostly marine, many species are estuarine, encountering very low salinities (e.g. <1), and a few members of the family live in true freshwater habitats. This family is subdivided into three sub-families (Nelson 1994): Batrachoidinae, which contains fifteen genera including the genus *Opsanus*, Porichthyinae, which contains the mid shipman genera Aphos and Porichthys and Thalassophryninae, which contains the venomous toadfish genera Daector and Thalassophryne (Collette 1966). At the present only limited molecular phylogenetic information is available for this fish group (Collette 2001).

Glutathione peroxidase

Aerobic reactions lead to the production of reactive oxygen species (ROS), which can be toxic to the cells. Biotic and abiotic stresses can trigger a dramatic increase in the generation of ROS in intracellular environment. In this context, aerobic organisms have developed several non-enzymatic and enzymatic systems to neutralize these compounds. The enzymatic systems include a set of gene products such as superoxide dismutases, catalases, ascorbate peroxidises and glutathione peroxidases (GPX) (Fink and Scandalios 2002). Glutathione peroxidase (EC 1.11.1.9 and EC1.11.1.12) is the general name for a family of multiple isozymes that catalyze the reduction of H_2O_2 or organic hydroperoxides to water or corresponding alcohols using reduced glutathione (GSH) as an electron donor (H_2O_2 + 2GSH → GS-SG + $2H_2O$) (Fig. 1).

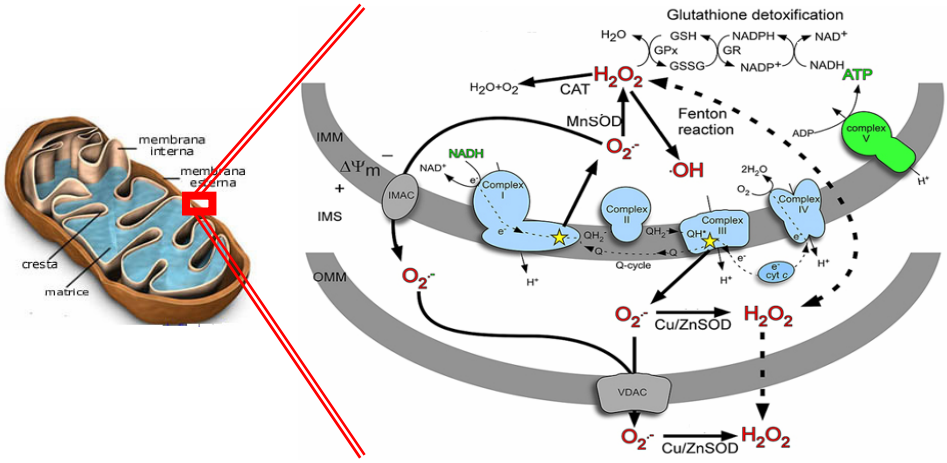


Figure 1: Picture of catalytic function of GPXs.

In Metazoa, some GPX have a selenocysteine and selenium-dependent glutathione peroxidase activity (Brown, Pickard et al. 2000). In mammalian tissues, there are four major selenium dependent GPx isozymes: (a) classical GPx (GPx1), which is found in red cells, liver, lung and kidney; (b) gastrointestinal GPx (GPx2); (c) plasma GPx (GPx3), which is present in different organs such as kidney, lung, epididymus, vas deferens, placenta, seminal vesicle, heart and muscle; (d) phospholipid GPx (PHGPx4 or GPx4),which is also broadly distributed in different tissues. GPx1, 2 and 3 are homotetramers, whereas GPx4 is functional as a monomer (Toppo, Vanin et al. 2008).

Two other isozymes, without Se-Cys, GPx5 and GPx6, had been found in mammalian. GPx5 is expressed in the epididymus as a secreted protein (Ghyselinck, Jimenez et al. 1989) and GPx6, was discovered as specifically expressed in the olfactory epithelium and previously named olfactory-metabolizing protein (OMP) (Dear, Campbell et al. 1991).

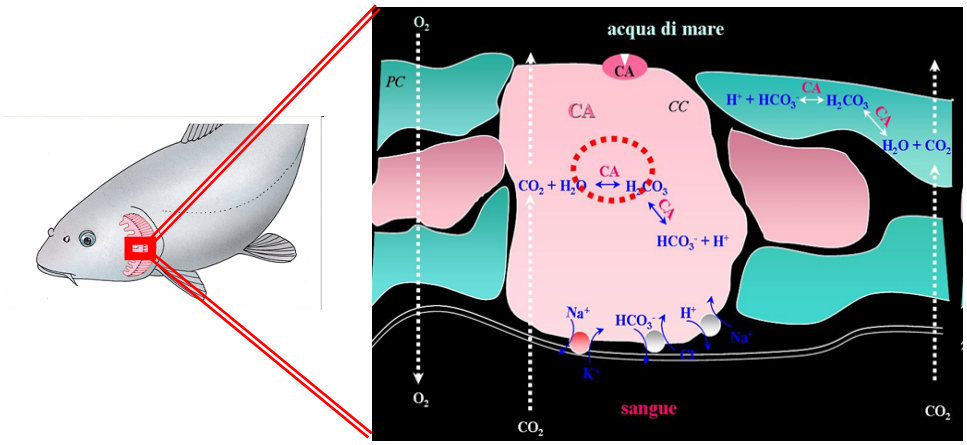
More recently, a new phospholipid hydroperoxide glutathione peroxidase (PHGPx) was described in mammals, which incorporates cysteine instead of selenocysteine in the conserved catalytic motif. PHGPx, also named NPGPx, corresponds to the seventh group of GPX found in mammals.

The evolutionary history of GPx genes is highly complex and is the result of a number of independent evolutionary events that might be in association with processes of functional convergence. It has been postulated that, in mammals, the GPX gene family would be evolved from a common ancestor gene by duplication events, followed by random mobilization in the genome. In addition, it is known that mammalian PHGPx must be considered as a phylogenetically ancient achievement of the GPx family. The analysis reveals that vertebrate GPx1, 2, 3, 5 and 6 may have had a common phylogenetic origin, probably distinct from GPx4. GPx gene families evolved by duplication from a common ancestral gene probably related to the PHGPx because sequences related to this group are also present in plants, arthropods and fungi (Margis, Dunand et al. 2008). After the first duplication event in vertebrates, this ancestor diverged to form two groups: the first evolved into GPx4 sequences, and the second group diverged subsequently to produce the GPx7, GPx1 and GPx2 group and the GPx3, GPx5 and GPx6 cluster. This suggests that the different GPx genes, corresponding to a protein with a distinct subcellular location, were generated by duplication events from a single nuclear ancestral gene (Margis, Dunand et al. 2008).

Besides are detailed informations on the evolution of hypoxia-tolerance physiology in many fish but the situation about hyperoxia-tolerant is somewhat more hopeful. This is because a larger amount of empirical as opposed to mechanistic information is available on hypoxia tolerance of different fish groups. At least as a first approximation, this appears consistent with multiple, independent origins of hypoxia-tolerance mechanisms in teleosts fish (Hochachka and Lutz 2001).

Carbonic anhydrase

Acid–base regulation in vertebrates is inextricably linked to carbon dioxide (CO_2) excretion through the reversible hydration/dehydration reactions of CO_2 and the acid–base equivalents H^+ and HCO^{3-}: $CO_2+H_2O \leftrightarrow H^+ + HCO_3^-$. Carbonic anhydrase is the enzyme that carries out this reaction. In fish, acid–base regulation is also coupled to ionic regulation, because acid–base compensation relies primarily on the direct transfer of H^+ and HCO_3^- across the gill in exchange for Na^+ and Cl^-, respectively (Fig. 2). Regulation of NaCl movement across the gill, in turn, is the keystone to maintaining ionic and osmotic balance in fish.



$$CO_2 + H_2O \leftrightarrows H_2CO_3 \leftrightarrows HCO_3^- + H^+$$

Figure 2: Picture of catalytic function of CAs.

CA (E.C. 4.2.1.1) is an ubiquitous enzyme and constitutes a family of zinc (Sly and Hu 1995)). In general, there are three distinct groups of CA isozymes within the α - CA gene family. One of these groups contains the cytoplasmic CAs, which includes mammalian CA I, II, III, V, VII and XIII. These isozymes have been found in the cytoplasm of various tissues, with the exception of the mitochondrial confined CA V. An other group of isozymes, named the membrane-bound CAs, consists of mammalian CAIV, IX, XII, XIV and XV. CA VIII, X and XI, named the CA-related proteins (CA-RP; (Tashian, Hewett-Emmett et al. 2000)). The various CA isozymes are found in many different tissues and are involved in a number of different physiological processes, including bone resorption, calcification, ion transport, acid–base transport, and a number of different metabolic processes.

The fish gill is a complex organ, knew to be involved in respiratory gas exchange, ion transport, and acid–base regulation. CA, abundantly present in gill epithelial cells, is assumed to play a role in these processes (Perry and Laurent 1990). Osmoregulating marine fish maintain extracellular fluid osmolality at 300–350 mosmol/kg and, as a consequence, experience continuous diffusive water loss to the hypertonic surrounding seawater (1000 mosmol/kg). To withstand this osmoregulatory challenge, fish drink seawater and ingested fluid is modified along the gastrointestinal tract, and the intestine plays a key role in marine fish osmoregulation absorbing NaCl and water (Marshall and Grosell 2005). The osmoregulatory role of the intestinal epithelium of marine teleost fish is the salt and water absorption to maintain water balance, whereas the gill extrudes salt absorbed from the intestine (Marshall and Grosell 2005). Considering the roles of gill and intestine CA in osmoregulatory processes, the relation between branchial CA activity and/or distribution and environmental salinity has been investigated in several species, yielding controversial results (Mashiter, 1975; Dimberg et al., 1981; Haswell et al., 1983; Lacy, 1983; Zbanyszek, 1984; Perry and Laurent, 1990; Flügel et al., 1991). In the flounder (*Platichthys flesus*), an euryhaline species, no significant differences in CA levels between seawater- and freshwater-adapted fish have been found in earlier studies in gills (Mashiter 1975) and red cells. No relation to salinity was reported for the CA activity in erythrocytes and gills of eel (*Anguilla anguilla*) (Haswell, Raffin et al. 1983). However, the esterase activity of CA in gills of young coho salmon (*Oncorhynchus kisutch*) was significantly higher in salt water adapted compared to freshwater fish (Zbanyszek 1984). The specific CA activity in gills of *Oreochromis mossambicus* increased in relation to an enhanced environmental salinity (Kültz et al., 1992; Sender et al., 1999). In killifish (*Fundulus heteroclitus*) expression of both Na^+/H^+-exchanger 2 and carbonic anhydrase 2 mRNA increased as early as 12 h after transfer in freshwater, so transcriptional regulation of these genes may also play a role in the early stages of freshwater acclimatisation (Scott, Claiborne et al. 2005). In the pufferfish (*Tetraodon nigroviridis*), Tang and co-workers (Tang and Lee 2007) compared the effect of environmental salinity on the protein expression of carbonic anhydrase (CAII) in teleost gills, as well as to examine interaction of CAII and anion freshwater rather than seawater individuals.

Other studies (Boutet, Long Ky et al. 2006), show that CAII mRNA level is higher in gill of saltwater- with respect to freshwater-acclimated sea bass (*Dicentrarchus*

labrax) and that the CA expression in gill (or gill and associated red blood cells) is higher in wild animals from higher salt concentrations. Earlier studies conducted on the rainbow trout showed a higher CA mRNA expression rate as well as higher enzyme activity in red blood cells than in all other studied tissues, such as kidney, gill or muscles (Esbaugh and Tufts, 2004; Boutet et al., 2006).

Phylogenetic analyses have shown that mammalian CAI, II and III evolved through a series of gene duplication events (Hewett-Emmett and Tashian 1991). In these studies, however, a high activity of CAII was likely to the ancestral CA, while CA III has low activity (Tashian 1992). In contrast, biochemical evidence of red blood cell CA isozymes from a modern representative of an ancient vertebrate lineage (agnathans) suggested that the low activity CAI isozyme was the ancestral state (Henry, Tufts et al. 1993). It is therefore likely that the ancestral cytoplasmic CA isozyme was a high activity enzyme, and the catalytic structure has been conserved throughout the vertebrate lineage. The structural components of the CA catalytic mechanism are remarkably conserved throughout vertebrates (Esbaugh and Tufts 2006).

Species studied

Antarctic species

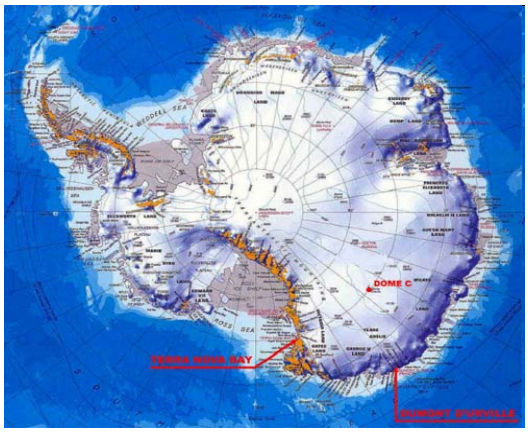


Figure 3: Antarctic continent.

The Southern Ocean that surrounding Antarctica is delimited by the Circumpolar Antarctic Current (Polar Front) that forms a natural barrier through for the crossing of small sized marine animals. In the area, between the Polar Front and the continental margin, the environment is characterized by great stability of physical

parameters, for example, temperature, salinity (with no freshwater inflows and only very minor salinity changes during the annual cycle of sea ice formation and melting) and oxygen concentration. The average annual temperature of water is -1.86 °C, the average annual salinity is 34.8 ppt and the average annual of dissolved oxygen concentration is 9 mg/l (Legg et al., 2009; Meiner et al., 2009).

The Antarctic teleosts were caught on Italian base at Terra Nova Bay, Ross Sea (74°42' S, 164°7' E) (Fig. 3).

Trematomus bernacchii (Boulanger, 1902)

Figure 4: *Trematomus bernacchii*

It is an Antarctic Notothenidae species that lives in Southern Ocean in a deep from 0 to 700 meters. It has a small head, close set eyes, general small scales and few tube scales in the lateral line. His body size range from 54 to 263 mm. He is well adapted to extremely low and stable temperatures (-1.86°C, annual mean temperature of McMurdo Sound) (Littlepage 1965). Spawning occurs in mid-October to early November. Eggs are demersal and attached to algae. It feeds on polychaetes, gastropods, isopods, amphipods and few algae. The habitat is among moraine rocks covered with algae (*Monostroma* and *Leptosarca* and below that *Desmarestia* and *Phyllogigas*). There is a nine month ice cover (Miller 1993).

Trematomus lepidorhinus (Poppenheim, 1911)

Figure 5: *Trematomus lepidorhinus*

He is an Antarctic Notothenidae species. It head is not a fully scaled and the vent is situated near to tip of snout, the eye diameter is short. The colour is brown with irregular and some what indistinct dark crossbars and spinous dorsal are black. Mouth and branchial cavities are black. The body size ranges from 29 to 280 mm. *Trematomus lepidorhinus* lives in the deep Ocean, from 200 to 800 meters. The distribution of this fish is surrounding Southern Ocean. It feeds on amphipods, copepods and polychaetes (Miller 1993).

***Trematomus eulepidotus* (Regan, 1914)**

Figure 6: *Trematomus eulepidotus*

It is an Antarctic Notothenidae species; it closely resembles *T. lepidorhinus* but differs in the shorter snout, broader interorbital region, more oblique mouth, shorter tail, more numerous dorsal rays, and much shorter middle lateral line. The colour is brownish with the dark spots connected to form a network and soft dorsal have oblique dark stripes. Body size is over 300 mm. It is epibenthic and lives in a range of deep from 70 to 650 m. He is common in water lower than 250 m, where there are more nutrients. It feed on nudibrancs, amphipods, copepods, polychaete, krill, crustacea, chetognata and fishes (Miller 1993).

***Cygnodraco mawsoni* (Waite, 1916)**

Figure 7: *Cygnodraco mawsoni*

It is a Bathydraconidae Antarctic species, the *sister group* closer to icefish (fish without haemoglobin). It is benthonic and lives in water from 110 to 330 m in depth. The body is yellowish or greyish-brown, traces of a dark line on each side of the snout, a dark spot below the eye, and a dark bar running backward from the eye toward the suboperculum. His size ranges from 125 to 466 mm. It feeds on benthic organisms and benhthopelagic like fishes belong to *Trematomus* species, decapoda, amphipods and polychetes (Miller 1993).

Intertidal species, Gazi Bay (Kenya)

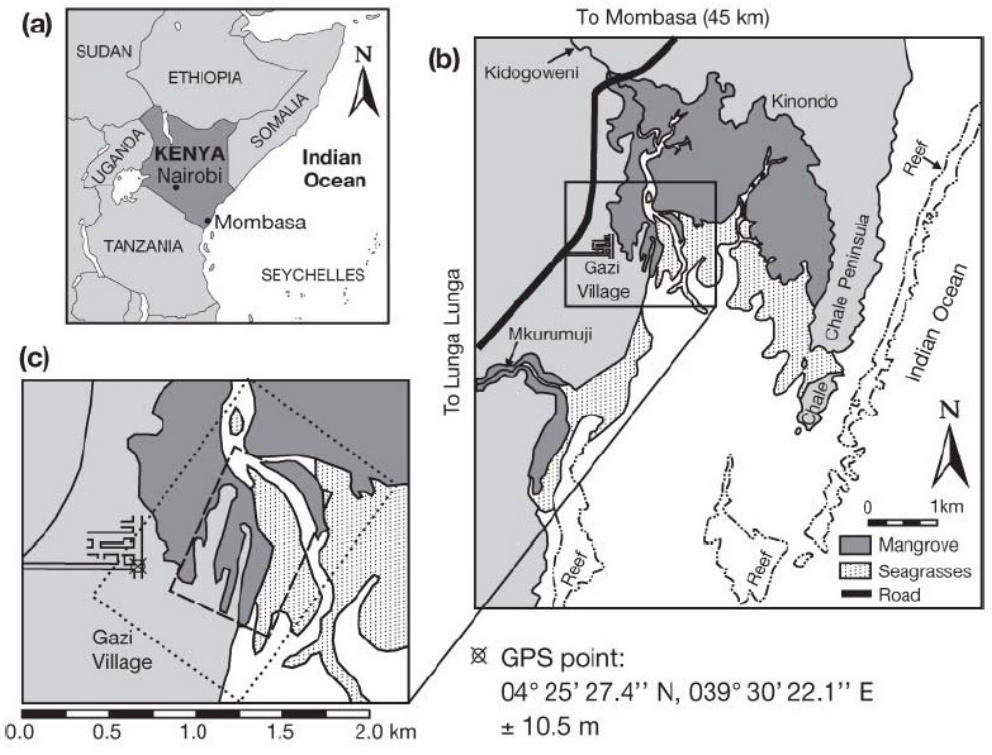


Figure 8: (a) Kenya. (b) Gazi Bay, showing areas of mangroves, seagrasses and coral reef. (c) Location of study site in Gazi.

Gazi Bay, Kenya, (4° 26' S, 39° 30' E) is located about 45 km south of Mombasa (Fig. 8). Fishes have been cough on a mangrove stretch adjacent to the village of Gazi (Fig. 8), where there are two seasonal rivers, Kidogoweni and Mkurumuji, that provide most of the overland freshwater input into the bay, but tidal influence (spring tidal amplitude is about 3.5 to 4 m) are more important.

Climatic data, available for Mombasa (Fig. 8), show an annual average temperature of 26.15°C (Dahdouh-Guebas, Van Pottelbergh et al. 2004). The average

annual salinity is 28.5 ppt recorded on river and 32.4 ppt recorded on coast. The average annual of dissolved oxygen concentration is 6.02 mg/l on coast (Schmitz, Jansen et al. 2007).

Periophtalmus sobrinus (Eggert, 1935)

Figure 9: *Periophtalmus sobrinus*

Periophtalmus sobrinus is a semi-terrestrial mudskipper fish that is strongly present in mangroves, building mud towers around their burrows. This species lives in the seaward edge of the mangroves, extending into the mangroves only where it is daily inundated. They are amphibious and can survive long periods out of water. The dark brown body size is ~14 cm length. It has blunt snout and dorsal fins with white margin and black band below. Pelvic fins are fused into an adhesive disc, when it is well developed. Spinous dorsal are present or absent; when they are present they have 2-8 flexible spines and they are discontinuous with soft dorsal. Typically nest spawners with non-spherical eggs guarded by the male. This species can be found in West Indian Ocean, Red Sea and Kenya coast. It feeds on small invertebrates and fish larvae (Richmond 1997).

Intertidal species, Florida Bay (Florida, USA)

Figure 10: Florida Bay, Florida

Florida Bay is a triangular-shaped shallow marine embayment located in a subtropical region. Morphologically, the Bay is dominated by an extensive system of shallow mud-banks and adjacent basins of relatively shallow depths. Florida Bay is bound to the north by the Everglades and receives freshwater input via several streams, nearly all located in the north eastern corner of Florida Bay (McIvor, Ley et al. 1994). To the west, a relatively open connection with the southwest Florida shelf is present, through which a large amount of physical forcing (e.g. wind and tidal) exchanges into Florida Bay (Wang, van de Kreeke et al. 1994). To the south, Florida Bay is bound by the Florida Keys; however, there is a limited exchange with the coastal Atlantic Ocean through tidal channels between the Keys (Smith, Tilman et al. 1999). The three primary sources of salinity variation in Florida Bay are precipitation and freshwater runoff (which decrease salinity) and evaporation (which increases salinity). In Florida Bay the hypersaline conditions prevail during early summer at the end of the dry season and estuarine conditions prevail in early winter at the end of the wet season (Kelble, Johns et al. 2007).

Opsanus beta (Goode and Bean, 1880)

Figure 11: *Opsanus beta*

Toadfish is scaleless, with eyes set high on large heads. A mouth is also large, with both maxilla and premaxilla, and decorated with barbels and skin flaps. It is drab in colour and the size ranges from 7.5 cm length, to 57 cm. The gills are small and occur only on the sides of the fish. The pelvic fins are forward of the pectoral fins, usually under the gills, and have one spine with several soft rays. This species is commonly found in seagrass beds and sandy and rock rubble bottoms located in bays, lagoons, and shallows coastal areas. It lives in Western Central Atlantic: Florida (USA), Little Bahama Bank (Bahamas) and the entire Gulf of Mexico to Campeche, Mexico. This bottom-dwelling, sluggish fish occurs as deep as 250 m. It buries itself in sand or hides among seaweeds, darting out to capture prey. A hardy fish, the gulf toadfish can remain alive for an extended period of time out of water as well as being able to survive in waters containing low levels of dissolved oxygen (Hutchins 1998).

Paper summary

Molecular and phylogenetic analysis of Antarctic fish Glutathione Peroxidase

Giovanna Sattin, Gianfranco Santovito, Arnaldo Cassini

Glutathione peroxidise (GPx) is an enzyme involved in a protection from oxidative stress damage cause by respiratory chain. It is closely linked to oxygen concentration and activity and expression change to variotion of oxygen concentration (Fink and Scandalios 2002). GPx catalyze the reduction of H_2O_2 or organic hydroperoxides to water or corresponding alcohols using reduced glutathione (GSH) as an electron donor (Brown, Pickard et al. 2000). It is well studied in mammalian where different isoforms are found and characterized (Margis, Dunand et al. 2008) but few notices are available for fish GPx. GPX is involved with hypoxia tolerance in different fish groups together in independent origins of hypoxia-tolerance mechanisms in teleosts (Hochachka and Lutz 2001) but nothing is know about hyperoxia tolerance. Consequently, the present paper focuses on cloning and analysis of phylogenetic reconstruction of teleosts GPx. The GPX was studied in Antarctic teleosts.

The Antarctic teleosts were choose for features of their habitat. They live in Ocean area delimited by Antarctica continent and the Polar Front. In this zone the environmental parameters are very stable, it is considered like a evolutionary microhabitat where fish occupied different ecological niches and developed particular adaptation (Eastman 2000). Many nototenioids fish that live in this environment are endemic (Eastman 1993; Stankovic, Spalik et al. 2002). The Antarctic Ocean forms a unique evolutionary site, suitable to study physiologic, morphologic and behavioural adaptation.

After RNA extraction from teleosts gills, the GPxs were cloned. The nucleotide sequences obtained were translates in aminoacidic sequences with an open reading frame of 191 aminoacids.

The sequences obtained were compared to other teleost GPx sequences available in GenBank. The similarities in nucleotides and aminoacids between teleosts are over 84%. The aminoacids involved in selenium fixation (Gln^{84} and Trp^{162}) (Ursini,

Maiorino et al. 1995), stabilization of the enzyme structure (Asn^{44}–Tyr^{56}; Leu^{74}–Gln^{84}; Trp^{162}–Phe^{164}) (Ursini, Maiorino et al. 1995) and catalytic center ($Arg^{54, 100,181}$ and Lys^{88}) (Aumann, Bedorf et al. 1997) are conserved in all teleosts species.

In all GPxs, there are three well conserved regions (Tosatto, Bosello et al. 2008), the first one is the region around SE-CYS, comprises from residue 41 to residue 62 ("LI(V/E)NVASL**U**GTT(V/T/A)RDYTQ(M/N)NEL"); the second one is around Gln^{84}, from 70 residue to 86 residue ("GL(V/M)(I/V)LG(A/V)PCNQFGH**Q**EN") and the third one is comprises from aminoacid 162 to 169 ("WNFEKFL(I/V)"). This sequences are conserved in all teleosts except for Met^{59} which is substituted with Val in trout and Glu^{34} that is substituted with Val in tuna, the Val^{72} that is Met in *T. eulepidotus,* the Ile^{73} with Val in anguilla, flounder and carp and of Val^{76} with Ala in zebrafish, carp and goldfish.

There are two aminoacid residues conserved only among Antarctic fish. Hydrophobic Ala^{21} in the Antarctic GPx in place of polar Ser^{21} and polar charged Arg^{130} in place of hydrophobic charged Lys^{130}.

Probably, the different enzymes of GPx have an common origin and they not follow a linear evolutionary history. Using NJ and MP methods with aminoacidic sequences, we obtained phylogenetic reconstruction. Antarctic teleost GPXs group with other teleosts GPx-1, separated from GPx-1 of mammalians and birds. They group together in clade close to tuna, trout and anguilla. *C. mawsoni* are sister groups of the Trematomus genus. In according with our results, phylogenetic studies performed using 12S and 16S rRNA place *Cygnodraco mawsoni* (Bathyraconidae family), as a sister group of *Trematomus* (Notothenidae family) (Bargelloni, Marcato et al. 2000).

Molecular phylogenetic analysis of Carbonic anhydrase in teleosts. Insights into molecular adaptations to temperatures

Giovanna Sattin, Stefano Marino, Luigi Bubacco, Gianfranco Santovito, Mariano Beltramini

Carbonic anhydrase is an multifunctional ubiquitary enzyme involved in catalitic hydratation/dehydratation of CO_2 (Geers and Gros, 2000; Esbaugh and Tufts, 2006).

It plays a key role in different mechanism but most importantly is in the acid/base balance and osmoregulation. In fish, almost 98% of plasma CO_2 is present as bicarbonate ion and, in addition to CO_2 transport and pH homeostasys, CA plays a fundamental role in osmoregulation in seawater as well as freshwater (Heisler, 1984; Perry and Laurent, 1990; Henry and Swenson, 2000). At the moment 16 different isoforms of the enzyme are know in mammalians but only few of these are know in. They differ in their kinetic properties, tissue distribution and subcellular localization (Esbaugh and Tufts 2006).

Mammalian cytoplasmic CAII diverged in two different isoforms in fish: CAb that is the cytoplasmic erythrocytary enzyme and CAc that is cytoplasmic not erythrocytary CA (Esbaugh and Tufts, 2006).

My work it's focused on CAs of Antarctic and intertidal teleosts. We know from precedent study the important role of CA in osmoregulation (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), then we choose Antarctic and intertidal fish for environmental parameters of habitat where they live.

In southern Ocean the salinity is very low (34,8 ppt) (Legg, Briegleb et al. 2009) in contrast to intertidal zone where it is very high (43 ppt) (Kelble, Johns et al. 2007). In order, the parameters are very stable during the year in Antarctic Ocean (Legge et al., 2009), while in intertidal zone the parameters fluctuate during the day (in Kenya where mudskipper lives) (Dahdouh-Guebas, Van Pottelbergh et al. 2004) and during the season (in Florida, where toadfish lives) (Kelble, Johns et al. 2007).

Four Antarctic teleost CA sequences (*Trematomus bernacchii, T. lepidorhinus, T. eulepidotus, Cygnodraco mawsoni*) and two intertidal teleost GPx (*Periophtalmus sobrinus* and *Opsanus beta*) were cloned and sequenced. Nucleotide sequences obtained from RNA extraction were translated to an open reading frame of 259 aminoacids. The sequenced CAs were compared with other CA teleosts sequences available in GeneBank . The identity found for the sequences of antarctic teleosts with the other species is rather high, with amino acid identities ranging from 74 to 85%.

The aminoacids important for proton shuttling (His^{64}, Ser^{65} and Phe^{66}/Leu^{66}); hydrophobic pocket adjacent to the Zinc ion (Trp^{209}, Val^{121}, Leu^{198}, Val^{143}) and hydrogen bonding network (Gln^{92} with His^{94}, Glu^{117} with His^{119}, and Asn^{243} with His^{96})

(Christianson and Alexander 1989; Krebs, Rana et al. 1993) are conserved in almost all sequences, with only a few substitution. Phe^{66} is substituted to Leu, Leu ^{202} is Cys and Tyr^{244} is Phe in *Periophthalmus sobrinus*. This strongly conservation suggests that the catalytic mechanism of CA is highly conserved throughout the fish lineage.

Sequences of *T. bernacchii, T. lepidorhinus, T. eulepidotus, C. mawsoni*, *P. sobrinus* and *O. beta* together with available sequences of fish and mammalian sequences were used to reconstruct the CA molecular phylogeny by Maximum Parsimony (MP), Neighbor Joining (NJ) and Maximum Likelihood (ML) methods. The topology of trees was the same using different methods. CA duplication event occurred at some point in the evolution of teleost fish, or a genome duplication event occurred at the origin of modern fishes, could account for the presence of two cytoplasmic CA isozymes: CAb and CAc, where CAb is more expressed in blood (Esbaugh and Tufts, 2004; Esbaugh et al., 2005; Lin et al., 2008) while CAc is more expressed in the gills (Esbaugh et al., 2005; Lin et al., 2008). The CA sequenced are cytoplasmic CA, mudskipper (*Periophtalmus sobrinus*) CA is erythrocytary cytoplasmic (CAb), while the others CA sequenced are cytoplasmic not erythrocytary (CAc).

The CAs Antarctic teleosts do not group together; *Cygnodraco mawsoni* and *Trematomus eulepidotus* seim to be the sister group of cluster with *T. bernacchii, T. lepidorhinus, Chionodraco hamatus, Oreochromis mossambicus* and *Pseudopleuronectes americanus* bootstrap value node is not well supported (50%).

Tree topology CA differs teleosts compared to Antarctic phylogeny made by 16SrRNA sequences (Verde, Parisi et al. 2004).

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

Giovanna Sattin, Edward M. Mager, Mariano Beltramini, Martin Grosell

In this paper we investigated the role of Carbonic anhydrase in a seawater Gulf toadfish (*Opsanus beta*) exposed to hypersalinity. *O. beta* lives in Florida Bay, a zone where salinity and temperature fluctuation are linked to dry and wet season. Salinity ranges from a minimum of 24.2 ppt in November to a maximum of 41.8 ppt in July (Kelble, Johns et al. 2007) with the average temperature of 25.8°C (Melesse,

Krishnaswamy et al. 2009). CA catalyse the hydration of CO2 providing H^+ and HCO_3^- at a high rate (Perry and Laurent 1990) that are exchanged 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). A number of studies have examined osmoregulation by toadfish in intestine (Grosell and Genz, 2006; Grosell and Taylor, 2007; Genz et al., 2008), kidney (McDonald and Grosell 2006) and gill (Evans 1982). Many studies report expression and activity of CA in freshwater fish acclimated at different salinities yielding contrasting results (Mashiter, 1975; Dimberg et al., 1981; Haswell et al., 1983; Lacy, 1983; Zbanyszek, 1984; Perry and Laurent, 1990; Flügel et al., 1991) but are not study about seawater fish exposed to hypersalinity. The aim of this project was to clone and sequence the toadfish (*Opsanus beta*) cytoplasmic CA from intestinal tissue and to compare it's expression and activity in several different tissues (gill, anterior, middle and posterior intestine and rectum) in fish acclimated to regular seawater (~40 ppt) and after exposition to 60 ppt for two weeks.

The cloned sequence is 1,827 nucleotides long with an open reading frame that predicts a 260 amino acid protein, showed 78% identity to the CA of *Oncorhynchus mykiss* and *Pseudopleuronectes americanus.*

Measures of expression and activity of intestinal and branchial CA revealed that in seawater acclimated fish gills showed higher expression than in the intestinal tissue. High mean expression levels in response to exposure to elevated salinity were observed in the middle and posterior intestine and in the rectum. CA enzyme activity in the cytosolic fraction of the gill and intestine tissues was significantly elevated in fish exposed to 60 ppt as was the case for all intestinal segments and the rectum. When considering total CA enzymatic activity, differences between control and 60 ppt fish were only observed in the anterior and middle intestine.

The results obtained on activity and expression suggest that this enzyme (in intestinal tissue as well as in gills) is important for the response to hypersalinity of 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. 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.

Comparing early mRNA expression and activity after two weeks of acclimation reveals a very different response to hypersalinity among tissues. My 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.

In gills of seawater acclimated toadfish, CA activity levels were similar to all intestinal segments, the 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. 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.

General conclusions

Glutathione Peroxidase (GPX) and Carbonic Anhydrase (CA) are two important enzymes involved in the resistance mechanisms of organisms. GPX is important for its protective role against reactive oxygen species while CA plays a multifunctional role to regulate different functions such as acid-base regulation and osmoregulation. Both enzymes are well studied in mammalian but less information is available for fish.

Our study is focused on sequencing and the phylogenetic analysis of GPX and CA in fish (Paper I and II). Four new GPX sequences from antarctic species are described. Six new CA sequences (from four antarctic species and from two intertidal species) are also described. Furthermore, the study of the activity and expression of CA in seawater teleosts exposed to hypersalinity demonstrated that this enzyme is up-regulated during an environmental osmotic stress (Paper III).

GPX (Paper I) sequences results to be well conserved among teleosts. The active site and the catalytic regions are almost always conserved between teleosts. The same is observed also for CA sequences (Paper II). In the latter case we can see just a few different amino acids in a region included within 10 Å, and the active site residues are conserved through all fish (Paper II). In both Antarctic GPX and CA we have found sequence positions with aminoacid residues different with respect to other teleosts but conserved in Antarctic fish. This may indicate that Antarctic fish developed a structure whose physico-chemical properties preserve the catalytic function in Antarctic habitat, like it has been reported for *Chionodraco hamatus* (Marino, Hayakawa et al. 2007).

Molecular phylogenetic analysis (Paper I and II) shows a resolved tree for both CA and GPX. The Antarctic species cluster in different ways in the two molecular phylogenetic trees. The main difference is that in CA tree, the enzymes from Antarctic species do not cluster together and the bootstrap values of the corresponding nodes do not allow drawing a firm conclusion. In contrast, sequences from Antarctic species group all together in the GPX tree and *Cygnodraco mawsoni* is the sister group of *Trematomus* species. If we compare our phylogenetic tree with the Antarctic teleosts tree obtained using complete gene sequences of the mitochondrial encoded 16S rRNA (Near et al., 2004; Verde et al., 2004), the topology obtained for GPx tree is more similar to that obtained using 16s rRNA .

In the last paper (Paper III) I studied the expression and activity of CA in toadfish exposed to hypersalinity for two weeks. Both activity and expression of CA became higher after experimental exposition; however, results reveal a very different response to hypersalinity among tissues. The mRNA expression increases in the posterior intestine and rectum but not in gills. On the other hand, the enzymatic activity increases the same in all tissues. We can conclude that more distal segments of the intestine have the greatest mRNA expression response to elevated salinity. The activity, after exposition of fish to hypersalinity, became higher in all analyzed tissues: gills and all intestine segments. We know from precedent studies (Marshall 2002) that CA is not traditionally implicated in gill salt transport by marine fish, however, our observations suggest that CA may also play a role in osmoregulation in hypersaline environments.

Molecular and phylogenetic analysis of Antarctic fish Glutathione peroxidase

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Glutathione peroxidases (GPX EC 1.11.1.9 and EC 1.11.1.12) catalyze the reduction of H2O2 or organic hydroperoxides to water or corresponding alcohols using reduced glutathione. Mammalian GPXs are well known but few data are available for teleost GPX. In the present study, the evolution of the teleost glutathione peroxidase gene family was investigated by comprehensive phylogenetic analysis using the improved number of Antarctic teleosts (Trematomus bernacchii, T. eulepidotus, T. lepidorhinus and Cygnodraco mawsoni) glutathione peroxidase. The GPX cloned are an open reading frame of 191 amino acid ant them catalytic centre are well conserved compare to the other GPX teleosts sequences available. The phylogenetic study show that glutathione peroxidase classes have originated from independent evolutionary events such as gene duplication, gene losses, and that the origin of fish GPX is monophyletic. Antarctic fish GPX cluster together, in according to molecular and morphological phylogenetic reconstruction of Antarctic teleosts.

Introduction

Aerobic reactions that happen by respiratory chain causes the production of reactive oxygen species (ROS), such as superoxide radicals, hydroxyl radicals and hydrogen peroxide, which can be toxic to the cells. Biotic and abiotic stresses can cause a dramatic increase in the ROS generation. The organisms have developed several enzymes (superoxide dismutases, catalases, ascorbate peroxidises and glutathione peroxidases) to neutralize these compounds (Fink and Scandalios 2002; Santovito, Piccinni et al. 2005).

Glutathione peroxidase (GPX) is the general name for a family of multiple isozymes that catalyze the reduction of H_2O_2 or organic hydroperoxides to water or corresponding alcohols using reduced glutathione (GSH) as an electron donor. Some GPX have a selenocysteine (Brown, Pickard et al. 2000).

Four major selenium dependent GPX isozymes are found in mammalian tissues: (a) classical GPX (GPX-1) (in red cells, liver, lung and kidney); (b) gastrointestinal GPX (GPX-2); (c) plasma GPX (GPX-3), which is present in kidney, lung, epididymus, vas deferens, placenta, seminal vesicle, heart and muscle) and (d) phospholipid GPx (PHGPx4 or GPX-4), distributed in various tissues.

Results from previous studies (Margis, Dunand et al. 2008) suggest that the evolutionary history of GPX genes is highly complex and that is the result of a number of independent evolutionary events that might be in association with processes of functional convergence. It has been postulated that, in mammals, the GPX gene family would have evolved from a common ancestral gene by duplication events, followed by random mobilization in the genome. The analysis reveals that vertebrate GPX-1, -2, -3, -5 and -6 may have had a common phylogenetic origin and they evolved by duplication from a common ancestral gene, probably distinct from GPX-4 (Margis, Dunand et al. 2008).

After first duplication events in vertebrates, GPXs diverged to form two groups: the first evolved into GPX-4, the second group subsequently diverged producing GPX-7, GPX-1 and GPX-2 group and GPX-3, GPX-5 and GPX-6 cluster (Margis, Dunand et al. 2008).

Several data are available about the involvement of GPXs in hypoxia tolerance in different fish groups (Hochachka and Lutz 2001). Our study was focused on evolution of hyperoxic tolerant fish.

Teleosts living in Antarctic sea are permanently exposed to low temperatures (-1.9 °C) and low salinity (34.8 ppt) (Legg, Briegleb et al. 2009). This environment is characterized by a great stability of physical parameters, for example, temperature, salinity and oxygen content, which is uniformly high because low temperatures increase gas solubility. In the Antarctic sea the great stability is due to the continent that in the early Miocene (25–22 million years ago) was subject to a series of tectonic and oceanographic events that created a well delimited biogeographic area. Afterwards, Antarctica gradually became isolated and colder (Anderson 1999).

In the beginning of the early Miocene (25–22 million years ago) the Antarctic shelf was subjected to a series of tectonic and oceanographic events that altered geography and faunal composition. Antarctica gradually became isolated and cold and the expansion of ice shelf led to destruction and disturbance of inshore habitat (Anderson 1999). Changes of habitat and trophic structure of the Antarctic ecosystem led to the extinction of many fish present in Eocene. Thus the diversity of the fauna have been reduced and becoming Antarctica isolated, new ecologically niches became available to other fish that were diversifying (notothenioids) or immigrating (liparids and zoarcids), developing cold-water ecosystem. Antarctic teleosts developed different physiological, morphological and behavioural adaptations. Examples are the acquisition of antifreeze glycopeptides (Chen et al., 1997; Cheng and DeVries, 2002), the loss of erythrocytes and haemoglobin (Cocca et al., 1995; Zhao et al., 1998; Detrich, 2000; Di Prisco et al., 2002) and the variable patterns of myoglobin expression in muscle tissues (Sidell et al., 1997; Moylan and Sidell, 2000) of white blooded channichthyids.

In the area delimited between the Polar Front and the continental margin there are 222 species included in19 families (Eastman, 1993; Clarke and Johnston, 1996;

Eastman, 2000). Notothenioids order is dominant, not only in terms of number of species, but also in abundance of specimen and biomass at (>90%). The eight notothenioid families encompass a total of 44 genera and 129 species, 101 Antarctic and 28 non-Antarctic (Bargelloni et al., 2000; Stankovic et al., 2002; Near et al., 2004). They are ecologically diverse, and have been hypothesized to represent an adaptive radiation in the coastal Antarctic regions of the Southern Ocean (Clarke and Johnston 1996). One of the most interesting notothenioid lineages is the Channichthyidae family, or icefish, containing 16 recognized species (Iwami and Kock, 1990; Eastman, 2000; La Mesa et al., 2002). Most notothenioids are endemic to the Southern Ocean with the greatest number of species distributed along the Antarctic Continental Shelf (Eastman 1993). A number of species are endemic to temperate non-Antarctic areas north of the Southern Ocean such Australia, Tasmania, southern New Zealand, southern South America, and the Falkland Islands (Eastman 1993; Stankovic, Spalik et al. 2002). Phylogenetic analyses performed on mt-rRNA sequences results in an hypothesis that the channichthyids are monophyletic and nested in a paraphyletic Bathydraconidae (Bargelloni, Marcato et al. 2000). Phylogenetic hypotheses inferred from morphology data places the Bathydraconidae as sister group of Channichthyidae (Iwami and Kock 1990; Balushkin 2000).

Materials and Methods

Experimental animals

Trematomus bernacchii (Boulenger, 1902), *T. eulepidotus* (Regan, 1914), *T. lepidorhinus* (Pappenheim, 1911), *Cygnodraco mawsoni* (Waite, 1916) were obtained from Terra Nova Bay, Ross Sea (74°42' S, 164°7' E). *T. eulepidotus* were sampled during 21st Italian PNRA Antarctica expeditions, *Cygnodraco mawsoni* during the 14th expedition, *T. lepidorhinus* during the 16th expedition and *T. bernacchii* during the 14th and 17th expedition. Fish were euthanized (tricaine methanesulfonate, MS-222; 0.2 g L^{-1}), their gills were excised and quickly placed into cryotubes and snap-frozen in liquid nitrogen and later stored at -80 °C.

Cloning of glutathione peroxidase

Extraction of total RNA from gills and intestine was performed using TRIzol®. Subsequently the RNA was loaded in gel electrophoresis to confirm the integrity of RNA and it was quantified by spectrophotometer. Reverse transcription was performed with ImProm-II™ Reverse Transcription System (Promega) using 1 μg of RNA and oligodT primer (Table I).

5' and 3' RACE

An initial fragment of GPX cDNA was obtained by RT-PCR. Using degenerate primers designed by alignment of multiple GPX nucleotide sequences available in the Swiss-Prot database: *Thunnus maccoyii* (ABO38817), *Oncorhynchus mykiss* (AAG30013), *Anguilla japonica* (ACN78878). PCR reactions was performed using a MyCycler™ thermal Cycler (BIO-RAD) with Go-Taq Polymerase (Promega) (5 U/μl) and the following cycling parameters: 25 cycles 94°C for 50 s, different temperatures depending the used primers for 50 s and 72°C for 1 min. The used primers are listed in Table I.

RACE reactions were carried out following the protocols provided with the 5'/3' RACE Kit, NucleoTrap® mRNA (Macherey-Nagel). Specific primers for RACE reactions (Table I) were designed from the sequence previously obtained. Touchdown PCR cycling conditions were as follows: thirty cycles of 94°C for 30 sec, different temperatures depending the used primers for 1 min and 72°C for 1 min and the last cycle for 72°C for 1 min. Products from all PCR reactions were gel-purified with Nucleospin® Extract II, cloned into the pGEM®-T Easy vector (Promega) and sequenced.

Molecular phylogenetic analysis

Phylogenetic studies were based on GPX amino acid sequences using the data of Antarctic teleosts and those of other species available in GenBank. Multiple alignments were performed using the Clustal W software (Thompson, Higgins et al. 1994). The analyzed data set includes the GPX amino acid sequences listed in Table II. Phylogenetic reconstructions were performed according to the Neighbor Joining (NJ) method (Saitou and Nei 1987) and Maximum Parsimony (MP) method (Felsenstein 1978). 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).

Table I: Sequences and melting temperatures of primers used for GPX cDNA sequencing of the different species.

Primer	Sequence (5'- 3')	T_m
Degenerate		
GPXfw1	AATGT(G+C)GCGTC(G+T)CT(C+T)TGAGG	72°C
GPXfw2	CCCTGCAA(C+T)CAGTTCGG	54°C
GPXfw2	CCCTGCAA(C+T)CAGTTCGG(G+A+C)CA	62°C
GPXfw3	CTGGAACTTTGA(A+G)AAGTTCCT	60°C
GPXfw4	ATGAACGAGCT(G+C)CA(C+T)	48°C
GPXfw5	CAAG(A+C)G(A+T+G+C)TACAGCAG	52°C
GPXre3	AGGAACTT(C+T)TCAAAGTTCCAG	72°C
GPXre5	CTGCTGTA(A+T+G+C)C(G+T)CTTG	52°C
GPXfw1b	CCCTGCAA(C+T)CAGTTCGG(G+A+C)CA	66°C
GPXfw2b	CTGGAACTTTGA(A+G)AAGTTCCT	56°C
Race 5'		
Race5' 1	TGATGGCCGAACTGGTTG	56°C
Race5' 2	GGAGATACTTCAGGGACACA	58°C
Race5' 3	TCCAGGAGCTGAAACTTG	54°C
Race 3'		
Race3' 1	CAAGCTGATCATGTGGAG	54°C
Race3' 2	GCCTGGAACTTTGAGAAGTT	54°C
Race3' 3	CCAGATGAACGAGCTCCAG	60°C
Race3' 4	CTACCATGGCAAGAAAGGCTA	62°C
T. eulepidotus		
GPX eulRe1	ACAGTGCAGCTGCAAACATTC	62°C
GPX eulRe2	TATTGGTCCCGAAACACAGC	60°C
Universal primers		
Abridged Anchor Primer	GGCCACGCGTCGACTAG TACGGGIIGGGIIGGGIIG	
Abridged Universal Amplification Primer	GGCCACGCGTCGACTAGTAC	
Anchor Oligo (dT)	ACCACGCGTATCGATGTCG	
Anchor	ACCACGCGTATCGATGTCGT$_9$	

Table II: Amino acid sequences of GPX (and their accession numbers in GenBank) used for phylogenetic reconstructions.

Species	GenBank accession number	Isoform
Homo sapiens	NP_000572	I
	NP_002074	II
	NP_002075	III
	CAA06463	V
	AF320068	VII
Mus musculus	NP_032186	I
	NP_109602	II
	NP_001077398	III
	NP_032188	IV-2
	NP_001032830	IV-1
	NP_034473	V
	AAH13526	VI
	AAH03228	VII
	NP_081403	VIII
	AAD41533	
Bos taurus	P00435	I
Gallus gallus	NP_001156704	III
Xenopus tropicalis	NP_001015740	I
	NP_988961	III
Taeniopygia guttata	NP_001130041	I
Danio rerio	NP_001007282	I
	NP_001131027	III
	NP_001007283	IV
	NP_001018337	VII
	NP_956516	VIII
Salmo salar	ACH86324	IV
	NP_001134361	VII
Oncorhynchus mykiss	AAG30013	I
	NP_001117997	II
Cyprinus carpio	ACR33821	IVa
	ACR33822	IVb
Sparus aurata	ABF70948	I
Anguilla japonica	ACN78879	IV
	ACN78878	I
Carassius auratus	ABO69566	I
	ABO36294	IV
Dentex dentex	ABD74628	I
Platichthys flesus	CAC27424	
Oplegnathus fasciatus	AAU44619	
Micropterus salmoides	ACJ67885	
Oryzias javanicus	ABG54485	
Hypophthalmichthys molitrix	ABU84810	
Hypophthalmichthys nobilis	ABU84812	
Ctenopharyngodon idella	ACF39780	
Oreochromis mossambicus	ACV93251	
Thunnus maccoyii	ABO38817	
Perca flavescens	ACQ99329	III

Results and discussion

Sequence analyses and multiple alignments

GPX cDNAs of four Antarctic teleost species were cloned. In all species the complete coding sequences is consistent of 576 bp with deduced amino acid sequence of 191 amino acids (Table III). The predicted molecular masses are about 21.50 kDa for all GPXs.

Table III: Molecular properties of the putative glutathione peroxidase cDNA and the deduced amino acid sequences.

	Nucleotide				Protein	
Name	cDNA (bp)	5'-UTR (bp)	ORF (bp)	3'-UTR (bp)	Deduced sequence length (aa)	Predicted molecular mass (kDa)
Trematomus bernacchii	946	56	576	314	191	21.53
Trematomus eulepidotus	945	56	576	694	191	21.57
Trematomus lepidorhinus	946	56	576	314	191	21.53
Cygnodraco mawsoni	950	60	576	313	191	21.59

Figure 1 shows multiple alignment of GPX sequences from different teleosts (Danio rerio, Carassius auratus, *Hypophthalmichthys molitrix,* Oncorhynchus mykiss, *Oplegnathus fasciatus, Oreochromis mossambicus, Platichthys flesus, Anguilla japonica*, Thunnus maccoyii), and from human, bovine and mouse GPX-1 sequences. All GPX cDNAs had a TGA codon that encodes selenocysteine (SECIS; U). Identities in nucleotides and amino acids among teleosts are over 84%. Identities between Antarctic GPXs and GPX-2 is over 79%, with other GPXs the similarity is lower: 45% with GPX-3, 34% with GPX-4, 30% with GPX-7, 25% with GPX-8.

Residues Gln84 and Trp162 are involved in fixing selenium and are conserved in all sequences. Also Arg54, Lys88, Arg 100 and Arg 181 residues that are located toward the catalytic centre (Aumann, Bedorf et al. 1997) are conserved in all GPXs.

Based on the protein structure, previously determined for bovine, GPX-1 has three loop structures that stabilize the enzyme. The first loop is Asn^{44}–Tyr^{56}, the second is Leu^{74}–Gln^{84}, and the third is Trp^{162}–Phe^{164} (Ursini, Maiorino et al. 1995). The amino acids that form these three loops are well conserved in all teleosts. Asn^{79} and Lys^{114}, that are also important for the activity of GPX-1 (Aumann, Bedorf et al. 1997), are also always conserved in teleost GPXs. Sequences show a three conserved amino acids in a motif "PG-G", from residue 101 to residue 104, in the functional helix and the extended central insertion (Fig. 1) belong to the tetrameric structure. Besides, there are other three well conserved regions (Tosatto, Bosello et al. 2008). The first one is the region around SECIS, comprised from residue 41 to residue 62, where we found the typical motif "L(V/I)VN(V/T)ASx(**C/U**)G(L/F)TxxxYxxLxxL" that in teleosts becomes "LI(V/E)NVASL**U**GTT(V/T/A)RDYTQ(M/N)NEL". This sequence is conserved in all teleosts except for trout where Met^{59} that is substituted by Val and for tuna where Glu^{43} that is substituted by Val.

The second conserved region is around Gln^{84}, from 70 residue to 86 residue. The typical motif is "G(L/F)x(V/I)L(G/A)FPCNQFxx**Q**EP" that in fish becomes "GL(V/M)(I/V)LG(A/V)PCNQFGH**Q**EN". The substitution of Val^{72} in T. eulepidotus with Met is conservative because the two amino acids are isofunctional. The substitution of Ile^{73} with Val in anguilla, flounder and carp and of Val^{76} with Ala in zebrafish, carp and goldfish is conservative too, because also in this case the amino acids are isofunctional.

The third conserved region, comprised from amino acids 162 to 169, have "**W**NFxxKFL(V/I)" as typical motif. This motif in teleosts is "WNFEKFL(I/V)", where Ile^{169} is always substituted by Val except in O. fasciatus.

There are some conserved amino acid residues that are only present in Antarctic fish. Hydrophobic Ser[21] in the Antarctic GPXs takes place of polar Ala[21] and polar charged Arg[130] takes place of hydrophobic charged Lys[130]. It is to note that this latter substitution is condivided with mammals.

This indicates that essential catalytic properties are similar among the GPXs. In particular, all these observations indicate that the catalytic triad residues, determined in bovine GPX-1 (Epp, Ladenstein et al. 1983)) are strictly conserved: SECIS[49], Gln[84] and Trp[162] are 100% conserved in all examined sequences. The Asn[163] is also strictly conserved: this residue is thought to play an important functional role, and its conservation exceeds 99% over all vertebrate sequences, redefining the catalytic triad as a tetrad (Fukuhara and Kageyama 2005).

We may suppose that the tertiary structure of Antarctic GPXs should be similar to the structure already characterized in bovine. These results suggest that the evolution of GPXs has maintained most part of the protein sequence, in reason of its catalytic properties.

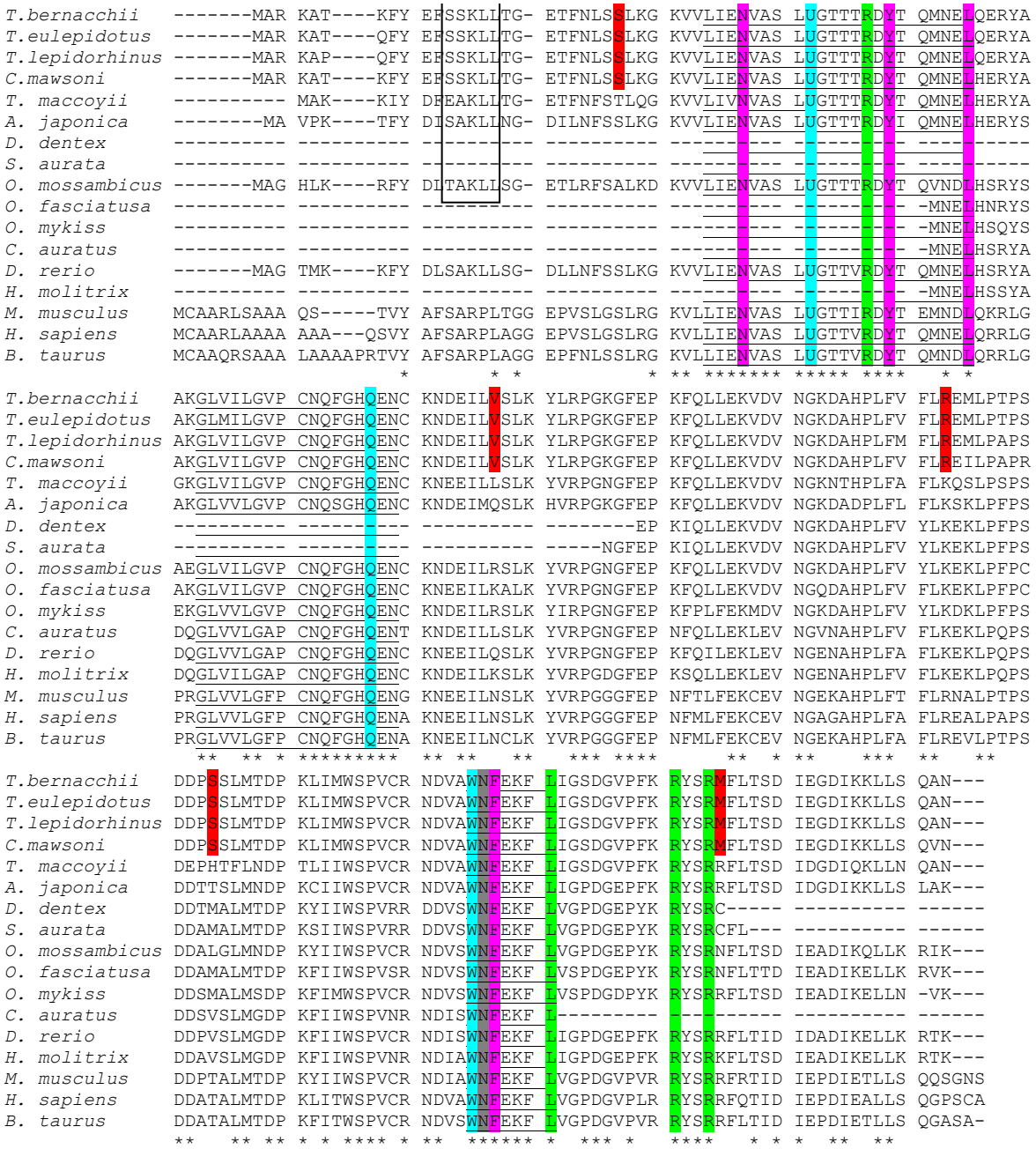

Figure. 1: Multiple sequence alignment of teleosts GPX-1, mouse, human and bovine GPX-1. The catalytic triad selenocysteine (U codified by TGA codon), glutamine, and tryptophan are blue highlighted. Amino acids conserved between sequences are marked by *. Sequences containing the "PG-G" motif characteristic of the tetrameric form are boxed. The amino acids

conserved only in Antarctic teleosts sequences are red highlighted. Arg^{54}, Lys^{88}, Arg ^{100} and Arg ^{181} that are located toward the catalytic centre are green highlighted. Three loops that are also important for the activity of GPX-1 (Asn^{44}–Tyr^{56}, Leu^{74}–Gln^{84}, Trp^{162}–Phe^{164}) are purple highlighted. are three well conserved regions ("LI(V/E)NVASLUGTT(V/T/A)RDYTQ(M/N) NEL", "GL(V/M)(I/V)LG(A/V)PCNQFGHQEN", "WNFEKFL(I/V)") are underlined. The Asn^{163} is grey highlighted.

Evolutionary relationships

The GPX gene family has an uncertain origin and does not follow a linear evolutionary history (Margis, Dunand et al. 2008). Different and independent evolutionary pathways seem to be operated in their members, making it difficult to establish an ancestral origin for many classes of GPX. It is possible that different isozymes possess a common origin; however, this is hard to determine that because their evolution is complex.

To investigate the evolutionary events that occurred in fish GPXs, phylogenetic analyses have been performed using all the know teleost GPXs amino acidic sequences, three mammalian GPXs, amphibian GPX and two bird GPXs. Tree reconstructions were obtained using different methods (NJ and MP). Figure 2 shows the tree obtained by NJ; anyway topology obtained by MP was very similar. The GPX-1 sequence of *Drosophila melanogaster* has been used as outgroup.

The tree highlights two main groups. The first group (clade A) is split into two other clusters, the first one characterized by the presence of mammalian, bird, amphibian and fish genes encoding for GPX-3,-5 and -6 and the second one characterized by the presence of mammalian, bird, and amphibian GPX-1,-2,-3 and fish GPX; the second (Clade B) grouped GPX-4, -8 and -7group.

The proteins of clade A, with the exception of mammalian GPX-5 and GPX-6, have a SECIS residue in the active site. This topology suggests that the common ancestor of this clade had an active side with selenocysteine, which have been later reverted to cysteine in GPX-5 and mouse/rat GPX-6. This conclusion is was also proposed by (Toppo, Vanin et al. 2008) studying evolutionary, structural, and sequence determinants of GPx specificity. The relationship among GPX-3, -5, and -6 are not resolved in both trees (MP and NJ) in reason of a low bootstrap value (<50) of GPX5/GPX6 branch. In this group, fish GPXs cluster outside tetraopod clade but the bootstrap value is quite low. The tree indicates also that GPX-5 and GPX-6 members are much more related to each other with respect to their GPX-3 relatives, making probable a recent duplication event (Fig.2). The Antarctic teleosts sequences are close to GPX-1s and they are grouped together and well separated from others of the sequence of *Cygnodraco mawsoni* is the sister group to the *Trematomus* sequences.According to our results, phylogenetic studies performed on 12S and 16S rRNA place *Cygnodraco mawsoni*, belong to Bathydraconidae family, like a sister group of *Trematomus,* belong in Notothenidae family (Bargelloni, Marcato et al. 2000).

It is to note that the Clade B shows two well-resolved subclades, one represented by GPX-4 and the other one represented by GPX-7. Proteins grouped in this clade are monomeric.

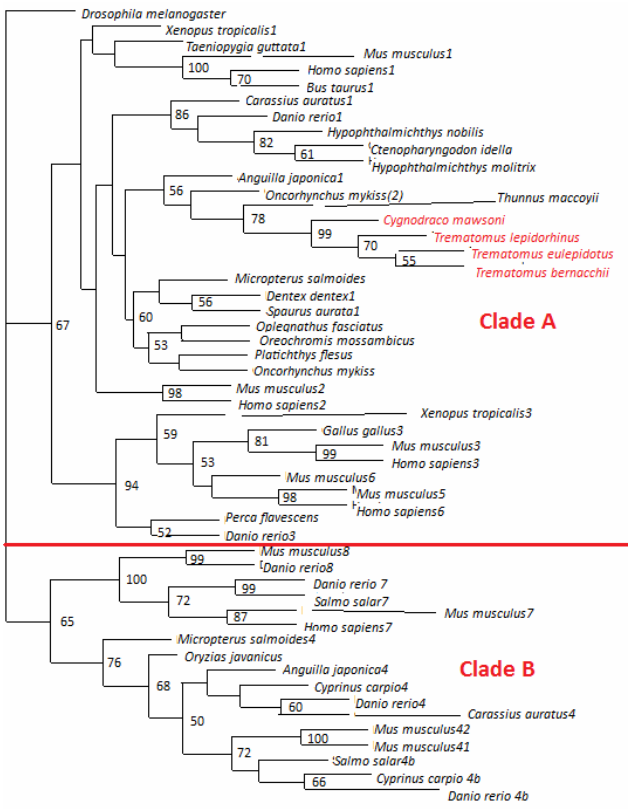

Figure 2: A phylogenetic analysis of the GPX isozymes. The phylogenetic tree was constructed using neighbour joining with support for nodes assessed using bootstrap analysis (1000 replicates), and ordered using drosophila GPX as an outgroup. Bootstrap values below 50 were not included, denoting poor branch support.

All these results allow us to hypothesise the scenario shown in figure 3. The ancestral *GPX* gene, codifying for a SECIS GPX, duplicated (D1) in two other ancestral *GPX* gene: 1 and 4. *GPX-1* duplicated (D2) in two other ancestral *GPXs*, *GPX-1* and *GPX-3*. *GPX-1* duplicated again (D4), only in mammalian, leading *GPX-1* and *GPX-2*. Fish do not express *GPX-2*. Only in trout there are a second GPX isoform, similar to GPX-1 that we propose to name GPX-1b, instead of GPX-2, as proposed by Goetz and co-worker (2004, unpublished). We can assume that these two genes resulted by a duplication event (D7) involving ancestral trout GPX-1.

About ancestral *GPX-3*, it duplicated (D5) only in mammalian leading ancestral *GPX-3* and ancestral *GPX-5*. *GPX-5* was subject to mutation that involved the SECIS codon, loosing the Se binding capacity. Thus, mammal GPX-5 and 6 have not Se-GPX activity. Except for D7 duplication, we suppose that the duplication event leading *GPX-3* and *GPX-5* ancestry is the most recent because involves

mammalian only. Afterwards, *GPX-5* duplicated in ancestral *GPX-5* and ancestral *GPX-6*.

Ancestral *GPX-4* duplicated (D3) in two genes: ancestral *GPX-4* and ancestral *GPX-7*. Ancestral *GPX-7* duplicated (D6) leading ancestral *GPX-7* and ancestral *GPX-8*. Ancestral *GPX-7* was subjected to mutation that involved the SECIS codon, leading to the second event of loss in Se-GPX activity of vertebrate GPX evolution history. Thus *GPX-7* and *GPX-8* are enzymes without SECIS. Other phylogenetic data suggest that also this second reversion to cysteine may be recent (Margis, Dunand et al. 2008).

This raises new questions, given that the cysteine-based enzymes are less efficient than the selenocysteine-based counterparts in countering oxidative stress. This could be an example of a novel direction taken by the GPX superfamily during evolution, adopting a different function and concomitantly possibly lacking any peroxidase activity.

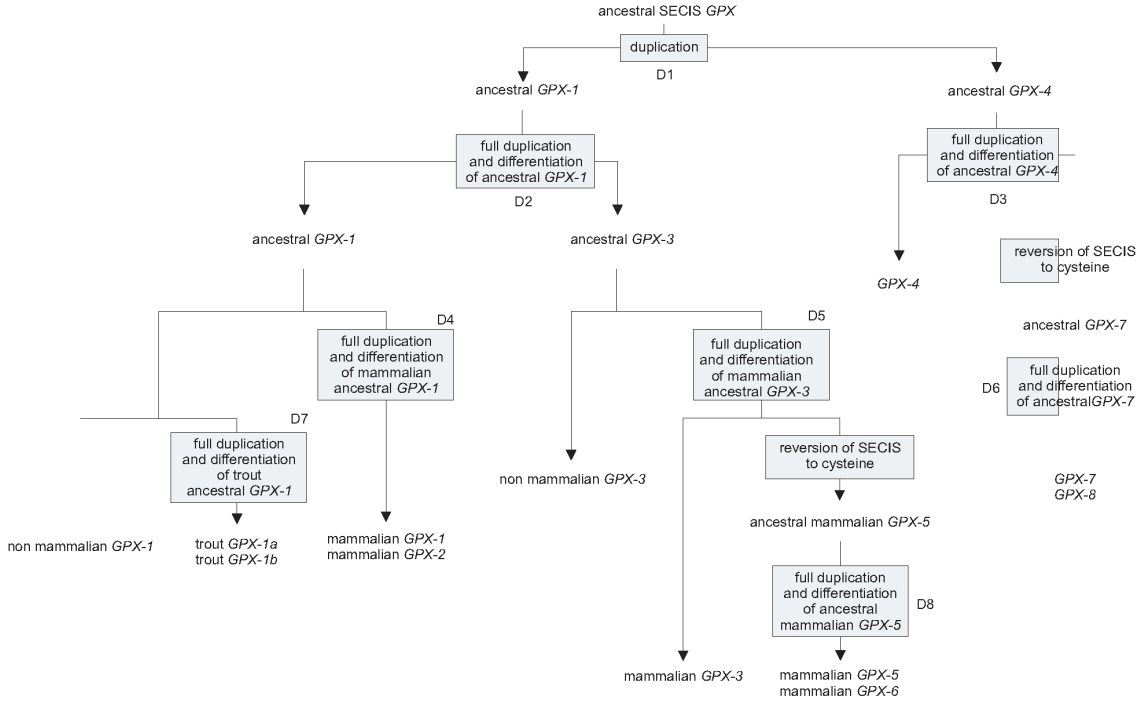

Figure 3: Reconstruction of GPX evolution. D= duplication event. R= reversion event.

Molecular phylogenetic analysis of Carbonic anhydrase in teleosts. Insights into the molecular adaptations to temperatures

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Carbonic anhydrase (CA) is a zinc metalloenzyme that catalyses the reversible reactions of CO_2 with water. In all vertebrates, CA plays a fundamental role in CO_2 transfer and excretion. To date, molecular analyses of the phylogeny of the α-CA gene family has been limited mostly to mammalian species with just a few sequences of temperate teleosts. In this work, we sequenced and analyzed CA of Antarctic fish species of nototenidae and bathydraconidae and of intertidal fish, mudskipper *Periophtalmus sobrinus* and toadfish *Opsanus beta*. The CAs with an open reading frame of 260 amino acids were cloned. The conservation of active site and catalytic centre were found by comparison with other sequences of temperate teleosts available in GeneBank. If we compare phylogenetic tree, obtained by morphological and molecular data, of Antarctic teleosts with CAs phylogenetic reconstruction we found that they are different, the Antarctic teleosts CAs don't group together.

Introduction

Carbonic anhydrase (CA, E.C. 4.2.1.1.: carbonate hydro-lyase) catalyzes the reversible reactions of hydration/dehydration of CO2 producing equivalents of H+ and HCO3–. This enzyme is found in many different tissues and appears to be involved in a number of physiological processes, including bone resumption, calcification, ion transport, acid–base transport and carbon dioxide transport (Geers and Gros, 2000; Esbaugh and Tufts, 2006).

Up to 16 different isozymes are known and divided in five different families α-ε (Tashian et al., 2000; Esbaugh and Tufts, 2006). They differ for their kinetic properties, tissue distribution and subcellular localization. The α-isoforms are found in mammals and three different groups are evident within the α-CA family: cytoplasmatic (I, II, III, V, VII and XIII), plasma membrane bound (IV, IX, XII, XIV, and XV) or secreted (VI). Furthermore, different CA-related proteins have been described (VIII, X, XI).

The catalytic activity of CA is due to the presence of a Zn ion coordinated by three N^{ε}-hystidine nitrogens in a tetrahedral complex. The fourth ligand of the metal is a hydroxide anion that represents the nucleophilic group interacting with the substrate CO_2. The generated bicarbonate anion is then exchanged with a water molecule that regenerates the Zn (II)-OH^-. The proton dissociated from the activated water molecule is released to the bulk solvent through a protein residue acting as a proton shuttle. While the first step of the catalytic reaction is nearly diffusion-limited, the release of the proton is rate limiting. However, it appears that there is not a unique way to accomplish this reaction. Hystidine residues are believed to be involved, such as His^{64} in CAII (Tu et al., 1989; Shimahara et al., 2007), although in the case of CAIII there are indications that the proton is released directly to the bulk solvent without the involvement of a shuttle residue (Jewell, Tu et al. 1991).

In all vertebrates, CA plays a fundamental role in CO_2 transfer and excretion. Erythrocyte CA catalyzes the hydration of metabolic CO_2, and HCO_3^- is exchanged with plasmatic Cl^-. At the respiratory epithelia, CO_2 is formed through the inverse reaction and passively diffuses in the ventilated medium (Henry and Swenson 2000). CA is involved also in pH homeostasis since the regulation of CO_2, HCO_3^- concentration affects also for H^+ concentration.

In fish, almost 98% of plasma CO_2 is present as bicarbonate and, in addition to CO_2 transport and pH homeostasis, CA plays a fundamental role in osmoregulation in seawater as well as freshwater (Heisler, 1984; Perry and Laurent, 1990; Henry and Swenson, 2000). Fish cytoplasmic CA is notably different from mammalian proteins. The current hypothesis is that it originated by divergence after the appearance of CAV and CAVII, which are present in both fish and mammals (Gilmour and Perry 2009). CAII in fish seems to represent a retained ancestral state, showing high activity, until appearance of teleosts, where CAII duplicated in two isoforms: CAb and CAc. CAb is expressed more in the blood of zebrafish (Lin, Liao et al. 2008) and trout (Esbaugh and Tufts, 2004; Esbaugh et al., 2005), whereas the second isoform, CAc, is more widely distributed, with high expression in the gills (Esbaugh et al., 2005; Lin et al., 2008).

Fish species occupy a great variety of ecological niches characterized by very different temperatures and salinities. In particular, fish that live in Antarctic sea are exposed to low temperatures and low salinity values (Legg, Briegleb et al. 2009). On annual basis, the temperature slightly changes around the average value of -1.9 °C and, due to the lack of freshwater inflows, minor salinity changes occur (average 34, 8 ppt) mostly related to the annual cycle of sea ice formation and melting. A characteristic feature of CA from Antarctic fish *Chionodracus hamatus* and *Trematomus bernacchii* resides in their higher activity at low temperatures, as compared to the temperate-water adapted species *Anguilla anguilla* (Maffia, Rizzello et al. 2001). This observation has been attributed to the modulation of the interaction between the catalytic water molecule and the Zn(II) ion as a function of the electrostatic potential distribution that results from amino acid sequence

and protein fold of the various proteins (Marino, Hayakawa et al. 2007).

The fish species studied in this work, *Trematomus bernacchii, Tr. eulepidotus, Tr. lepidorhinus* and *Cygnodraco mawsoni*, belong to the notothenioids family, the most represented fauna in terms of diversity, abundance and biomass in Southern Ocean (Eastman 1993). This clade exhibits adaptations related to metabolic functions and freezing avoidance in subzero Antarctic waters. They developed antifreeze glycopeptides, a key innovation in notothenioids (Chen et al., 1997; Cheng and DeVries, 2002), and is characterized by a high degree of morphological diversity (Kock 1992; Eastman 2005). The notothenioids are the endemism of Antarctic fish and it has been hypothesized that they represent an adaptive radiation in the coastal Antarctic regions of the Southern Ocean (Briggs 1974; Briggs 1996; Clarke and Johnston 1996). The eight notothenioid families now encompass a total of 44 genera and 129 species, 101 Antarctic and 28 non-Antarctic (Bargelloni et al., 2000; Stankovic et al., 2002; Near et al., 2004). The most important family of Notothenioid are the nototheniids, channichthyids, bathydraconids and artedidraconids, (Kock 1992).

Many morphological and molecular studies investigate the phylogenetic relationships between Antarctic fish (Chen, DeVries et al. 1997; Bargelloni, Marcato et al. 2000; Near, Pesavento et al. 2003; Near, Pesavento et al. 2004). A number of species are endemic to temperate non-Antarctic areas north of the Southern Ocean such as southern Australia, Tasmania, southern New Zealand, southern South America, and the Falkland Islands. The composition of the non-Antarctic notothenioid fauna includes both phylogenetically basal lineages, and species from clades that are hypothesized to have Antarctic origins but have dispersed to non-Antarctic regions of the Southern Ocean subsequent to the continental separation of Antarctica from Australia, New Zealand, and South America (Eastman 1993; Stankovic, Spalik et al. 2002). Most channichthyid species are confined to the Antarctic region, but at least three species are found outside of this region in the Kerguelen Islands and the Falkland Islands (Iwami and Kock 1990).

In contrast to the stability of physical parameters of Antarctic seawaters, mangrove areas are located at the interface between land and sea and thus are influenced by both terrestrial and marine factors. They are very unstable habitats where temperature, salinity and dissolved oxygen fluctuate largely (Lowe-McConell 1987; Morton 1989; Blaber 1997). High salinity, extreme tides, strong winds, high temperatures, muddy and anaerobic soils are the typical condition of this environment. Even in mangroves with strong river input, the combined effects of evaporation and transpiration may remove most of the freshwater entering the high salinity system at the end of dry season (Kathiresan 2001). Many intertidal fish, that are able to withstand these environmental changes, show morphological, physiological and behavioural adaptations which enable them to survive and reproduce in a habitat subject to regular changes (Lewis 1970; Bridges 1993; Gibson 1996). Among them, Mudskippers are a group of unusual amphibious fish (Perciformes, Gobiidae, and Oxudercinae) that are characteristic residents of many mangroves. They are euryhaline and highly adaptable to different environmental conditions, including salinity changes (Chew, Lim et al. 1990), aerial exposure (Kock, Lim et al. 1998), hypoxia (Chew and Ip 1990; Chew, Lim et al. 1990), and exposure to ammonia (Peng, Chew et al. 1998; Randall, Wilson et al. 1999; Ip, Randall et al. 2004). The mudskipper *Periophthalmodon schlosseri* is able to actively eliminate ammonia against large inward gradients (Randall, Wilson et al. 1999) and is highly tolerant to environmental ammonia (Peng, Chew et al. 1998). Wilson and co-workers (Wilson and Grosell 2003) investigated the role of carbonic anhydrase in ammonia elimination in this species demonstrating that intracellular CO_2 hydration is important to provide H^+ for NH_3 protonation to maintain P_{NH}´ gradients across the basolateral membrane and to provide NH_4^+ for apical Na^+/NH_4^+ exchange. The principal organs involved in acid-base regulation in mudskipper are gills, intestine (Grosell 2001), and kidneys (Gilmour and Perry 2009). The gills contain very high levels of CA that participates to acid-base regulation (Randall and Tsui 2006) together with NHE3 transporters in the apical membrane as well as the NBC1 and NKA that are found in the basolateral membrane of the cells (Hirata, Kaneko et al. 2003).

In this work we have sequenced CAs from cDNA obtained from four Antarctic fish species: three of the genus *Trematomus* (*T. eulepidotus, T. lepidorhinus, and T. bernacchii*) and one of the genus Cygnodraco (*C. mawsoni*). Our work is aimed at finding, by sequence comparison, the amino acid sequence motives characteristic of Antarctic fish species. The study has been extended to the CA of the mangrove fish *Periophthalmus sobrinus*, as an example of species adapted to high temperature and salinity environment.

Materials and Methods

Experimental animals

Trematomus bernacchii (Boulenger, 1902), *T. eulepidotus* (Regan, 1914), *T. lepidorhinus* (Pappenheim, 1911), *Cygnodraco mawsoni* (Waite, 1916) were obtained as by-catch from Baia Terra Nova, Ross Sea (74°42' S, 164°7' E). *T. eulepidotus* were sampled during XXI Italian campaign in Antarctica, *Cygnodraco mawsoni* during the XIV campaign, *T. lepidorhinus* during the XVII campaign and *T. bernacchii* during the XIV and XVII campaign. Mudskipper *Periophthalmus sobrinus* (Eggert, 1935) were caught from Gazy Bay, Kenya. Fish were euthanized (tricaine methanesulfonate, MS-222; 0.2 g L^{-1}), their gills were excised and quickly placed into cryotubes and snap-frozen in liquid nitrogen and later stored at -80 °C.

Cloning of cytoplasmic carbonic anhydrase

Extraction of total RNA was performed using TRIzol®. Subsequently the RNA was loaded in gel electrophoresis to confirm the integrity of RNA was maintained and it was quantified by spectrophotometer. Reverse transcription was performed with ImProm-II™ Reverse Transcription System (Promega) using 1 µg of RNA and oligodT primer (Table I).

Table I: Sequences and melting temperatures of primers used for sequencing CAs of the different species.

Table I: Sequences and melting temperatures of primers used for sequencing CAs of the different species.

Primer	Sequence (5'- 3')	T_m
Degenerate		
QSPIDI (R)*	CAGTCTCC(A+C+T)AT(A+T)GA(C+T)AT	48°C
QFH (F)	CAGTT(C+T)CA(C+T)TTCCA(C+T)TGGGG	64°C
Anchor (R)	ACCACGCGTATCGATGTCG	67°C
Anchor-dT (R)	ACCACGCGTATCGATGTCGT$_{(9)}$	75°C
ReCA (R)	AC(A+G)ATCCAGGTGAC(A+G)CTCTC	55°C
Periophtalmus		
Race5' 1	GTTTCACTTCCATTGGGGCA	60°C
Race5' 2	CTCTGAGCACACTGTGAAT	56°C
Race5' 3	GTGCACTGGAACACTAAGTA	58°C
Race3' 1	TCAGGGCCAAAGGCAAGCAGAC	70°C
Race3' 2	AGACCTCTTTCGCTGACTTCGAC	70°C
Race3' 3	GACCTCTTTCGCTGACTTCGACC	72°C
T. bernacchii		
Race5' 1	GTATTCCTCAAGATCGGTG	56°C
Race5' 2	CCAAAGGCAAGCAGACC	54°C
Race5' 3	GCTGACTTCGACCCATAG	58°C
T. eulepidotus		
Race5' 1	CCCCAATGGAAATGAAACTG	58°C
Race5' 2	GGGTATTTGGTGTTCCAGTG	60°C
Race5' 3	CCCACCACTGCAAGGCCA	60°C
Cygnodraco		
Cygnrev1	GGGTACTT(A+C)GTGTTCCAGTG	61°C
Cygnrev2	TCCAGGTGACACTCTCCAG	60°C
Cygnfor1	ACTGGGGAGCCTCTGATG	58°C
Cygnfor2	TGAGCTTCATTTGGTGCACTG	62°C
Race5' 1	TACGATGTCAATGGGAGACTG	62°C
Race5' 2	GAATGTCCGTTGTTGAGAATG	60°C
Race5' 3	CAGTCAGAGTTGAGCTGTC	58°C

5' and 3' RACE

To obtain sequence for RACE primer design, an initial fragment of CAII was obtained by PCR of fish gill cDNA. For this, degenerate primers were derived from alignment of multiple CAII nucleotide sequences available in the Swiss-Prot database: *Danio rerio* (NM_199215.1), *Oncorhynchus mykiss* (NM_001124221.1), *Tribolodon hakonensis* (AB055617.1). Reactions were performed using a MyCycler™ thermal Cycler (BIO-RAD) with Go-Taq Polymerase (Promega) (5 U/µl) using the following cycling parameters: 95°C for 50 s, different temperatures depending the primers used for 50 s and 72°C for 1 min. The primers used are shown in Table I.

RACE reactions were carried out following the protocols provided with the 5'/3' RACE Kit, 2nd Generation (Roche Applied Science). Primers for RACE reactions (Table 1) were designed from a fish specific sequence obtained from the degenerate PCR reaction. Touchdown PCR cycling conditions were as follows: five cycles of 94°C for 2min, 94°C for 15 sec, followed by 10 cycles 55°C for 30 sec, followed by 25 cycles (elongate each successive cycle by additional 20 sec) of 94°C for 15s, 55°C for 30s and 72°C for 40 sec and the last cycle for 72°C for 4 min. Products from all PCR reactions were gel-purified with GenElute™ PCR Clean-Up Kit or GenElute™ Gel Extraction Kit (Sigma), cloned into the pGEM®-T Easy vector (Promega) and sequenced.

Molecular phylogenetic analysis

Phylogenetic studies were based on amino acid sequences available from GeneBank. Multiple alignments were performed using the Clustal W software (Thompson, Higgins et al. 1994). The analyzed data set includes the CA amino acid sequences listed in Table II. The CA sequence of *Platichthys flesus* have been used for sequences compare but not for phylogenetic analysis because the aminoacidic sequence, in the middle of ORF, is not conserve. The sequence is questionable, especially, because one of hystidine that coordinates zinc binding ligand (His^{119}) is not conserved. 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).

Table II: Amino acid sequences of CAs (and their accession numbers in GenBank) used for phylogenetic reconstruction and sequences compare.

Species	GenBank accession number	Isoform
Homo sapiens	NP_001122303	I
	NP_000058	II
	NP_005172.1	III
	BC069649	IV
	NP_001730	Va
	NP_009151	Vb
	AAH33865	VII
	AAH14950	IX
	BC023981	XII
	AAH34412	XIV
Mus musculus	NP_001077426	I
	NP_033931	II
	NP_031632	III
	NP_031633.1	IV
	NP_031634	Va
	NP_851832	Vb
	NP_062386	
	NP_444300	VII
	NP_647466.2	IX
	NM_178396	XII
	AAH46995	XIV
	AAK16671.1	XV
Gallus gallus	P07630	II
Xenopus tropicalis	NP_001015729	II
	NM_001110051.1	XIV
Xenopus laevis	NP_001084717	IV
Petromyzon marinus	AAZ83742	
Danio rerio	CAM13156	II (CAc)
	NP_954685	II (CAb)
	NP_957107	VII
Squalus acanthias	AAZ03744	IV
	BAD36835	I
Oncorhynchus mykiss	NP_001117693	II
	NP_001117959	IV
Lepisosteus osseus	AAM94169	
Tribolodon hakonensis	BAB83090	
Cyprinus carpio	AAZ83743	II
Opsanus beta	ACU30149	II
Drosophila melanogaster	NP_648555	
Pseudopleuronectes americanus	AAV97962	
Platichthys flesus	AAC64172	
Chionodraco hamatus	P83299	I
Oreochromis mossambicus	AAQ89896	
Fundulus heteroclitus	AAX20367	II
Salmo salar	ACN10477	
	ACN10842	Vb
	ACI33869	
Tetraodon nigroviridis	CAF99720	

	CAG07521	VI
Dicentrarchus labrax	CAH69488	
Tribolodon hakonensis	BAB83090	II

Results

Sequence and Phylogenetic analysis

Isolation and sequencing of the cDNA obtained from gills of Antarctic fish (*Trematomus bernacchii, T. eulepidotus, T. lepidorhinus and Cygnodraco mawsoni*) and of the mudskipper (*Periophtalmus sobrinus*) yielded a complete 5' and 3' UTR sequences (Table III). The complete coding sequences consist in all species of 777 bp, coding for proteins of 259 amino acids in length. The predicted molecular masses are ~28 kDa. The features of nucleotide and deduced amino acid sequences are reported in Table III.

Table III: Molecular properties of the putative carbonic anhydrase cDNA and the deduced amino acid sequences.

Name	Accession number	Nucleotide cDNA (bp)	5'-UTR (bp)	ORF (bp)	3'-UTR (bp)	Deduced amino Acid sequence (aa)	Protein Native Protein (aa)	Predicted molecular mass (kDa)
Trematomus bernacchii	GQ443602*	1062	73	777	215	654	259	28.25
Trematomus eulepidotus	GQ443600*	1563	90	777	694	521	259	28.63
Trematomus lepidorhinus	GQ443601*	1656	175	777	704	552	259	28.45
Cygnodraco mawsoni		713	-	713	-	-	239	-
Periophthalmus sobrinus	GQ443603*	1217	272	777	168	406	259	28.45

*Those sequences will be available from September 2010.

Table IV: Amino acid sequences of teleosts CA II. Comparison of the amino acid residues located within 10 Å of the zinc ion, based on the reconstruction of (Marino, Hayakawa et al. 2007). The antarctic species are underlined. The different aminoacids are underlined and are in bold font.

Position number	5	7	16	29	30	62	64	65	66	67	92	93	94	95	96	97	104	105	106	107	116	117
proton shuttling ligand						#	#	#		#												
zinc binding ligand													*		*							
Trematomus bernacchii	W	Y	W	S	P	N	H	S	F	Q	Q	F	H	F	H	W	G	S	E	H	A	E
Trematomus lepidorhinus	W	Y	W	S	P	N	H	S	F	Q	Q	F	H	F	H	W	G	S	E	H	***F***	E
Trematomus eulepidotus	W	Y	W	S	P	N	H	S	F	Q	Q	F	H	F	H	W	G	S	E	H	**C**	E
Cygnodraco mawsoni	W	Y	W	S	P	N	H	S	F	Q	Q	F	H	F	H	W	G	S	E	H	**C**	E
Chionodraco hamatus	W	Y	W	S	P	N	H	S	F	Q	Q	F	H	F	H	W	G	S	E	H	A	E
Periophtalmus sobrunus	W	Y	W	S	P	N	H	S	**L**	Q	Q	F	H	F	H	W	G	S	E	H	**C**	E
Opsanus beta	W	Y	W	S	P	N	H	S	F	Q	Q	F	H	F	H	W	G	S	E	H	**C**	E
Lepisosteus osseus	W	Y	W	S	P	N	H	S	F	Q	Q	F	H	F	H	W	G	S	E	H	A	E
Tribolodon hakonensis	W	Y	W	S	P	N	H	S	F	Q	Q	F	H	F	H	W	G	S	E	H	A	E
Danio rerio (CAc)	W	Y	W	S	P	N	H	S	F	Q	Q	F	H	F	H	W	G	S	E	H	A	E
Danio rerio (CAb)	W	Y	W	S	P	N	H	S	F	Q	Q	F	H	F	H	W	G	S	E	H	**C**	E
Pseudopleuronectes americanus	W	Y	W	S	P	N	H	S	F	Q	Q	F	H	F	H	W	G	S	E	H	A	E
Platichthys flesus	W	Y	W	S	P	N	H	S	F	Q	Q	F	H	F	H	W	G	S	E	H	***L***	E
Oreochromis mossambicus	W	Y	W	S	P	N	H	S	F	Q	Q	F	H	F	H	W	G	S	E	H	A	E
Oncorhynchus mykiss (CAc)	W	Y	W	S	P	N	H	S	F	Q	Q	F	H	F	H	W	G	S	E	H	A	E
Oncorhynchus mykiss (CAb)	W	Y	W	S	P	N	H	S	F	Q	Q	F	H	F	H	W	G	S	E	H	**C**	E
Ciprinus carpio	W	Y	W	S	P	N	H	S	F	Q	Q	F	H	F	H	W	G	S	E	H	**C**	E
Salmo salar	W	Y	W	S	P	N	H	S	F	Q	Q	F	H	F	H	W	G	S	E	H	**C**	E
Fundulus heteroclitus	W	Y	W	S	P	N	H	S	F	Q	Q	F	H	F	H	W	G	S	E	H	A	E

Position number	118	119	120	121	140	142	143	144	145	196	197	198	199	200	202	206	208	209	243	244	245
zinc binding ligand		*																			
Trematomus bernacchii	L	H	L	V	L	V	V	G	V	S	L	T	T	P	L	V	W	I	N	Y	R
Trematomus lepidorhinus	L	H	L	V	L	V	V	G	V	S	L	T	T	P	L	V	W	I	N	Y	R
Trematomus eulepidotus	L	H	L	V	L	V	V	G	V	S	L	T	T	P	L	V	W	I	N	Y	R
Cygnodraco mawsoni	L	H	L	V	L	V	V	G	V	**P**	L	T	T	P	L	V	W	I	N	Y	R
Chionodraco hamatus	L	H	L	V	L	V	V	G	V	S	L	T	T	P	L	V	W	I	N	Y	R
Periophtalmus sobrunus	L	H	L	V	L	V	V	G	V	S	L	T	T	P	**C**	V	W	I	N	**F**	R
Opsanus beta	L	H	L	V	L	V	V	G	V	S	L	T	T	P	L	V	W	I	N	Y	R
Lepisosteus osseus	L	H	L	V	L	V	V	G	V	S	L	T	T	P	L	V	W	I	N	Y	R
Tribolodon hakonensis	L	H	L	V	L	V	V	G	V	S	L	T	T	P	L	V	W	I	N	Y	R
Danio rerio (CAc)	L	H	L	V	L	V	V	G	**I**	S	L	T	T	P	L	V	W	I	N	Y	R
Danio rerio (CAb)	L	H	L	V	L	V	V	G	V	S	L	T	T	P	L	V	W	I	N	Y	R
Pseudopleuronectes americanus	L	H	L	V	L	V	V	G	V	S	L	T	T	P	L	V	W	I	N	Y	R
Platichthys flesus	**C**	**T**	**G**	**T**	**P**	**S**	**L**	**S**	**E**	**G**	**P**	**L**	**P**	L	**W**	**P**	**L**	**S**	**K**	Y	R
Oreochromis mossambicus	L	H	L	V	L	V	V	G	V	S	L	T	T	P	L	V	W	I	N	Y	R
Oncorhynchus mykiss (CAc)	L	H	L	V	L	V	V	G	V	S	L	T	T	P	L	V	W	I	N	Y	R
Oncorhynchus mykiss (CAb)	L	H	L	V	L	V	V	G	V	S	L	T	T	P	L	V	W	I	N	Y	R
Ciprinus carpio	L	H	L	V	L	V	V	G	V	S	L	T	T	P	L	V	W	I	N	Y	R
Salmo salar	L	H	L	V	L	V	V	G	V	S	L	T	T	P	L	V	W	I	N	Y	R
Fundulus heteroclitus	L	H	L	V	L	V	V	G	V	S	L	T	T	P	L	V	W	I	N	Y	R

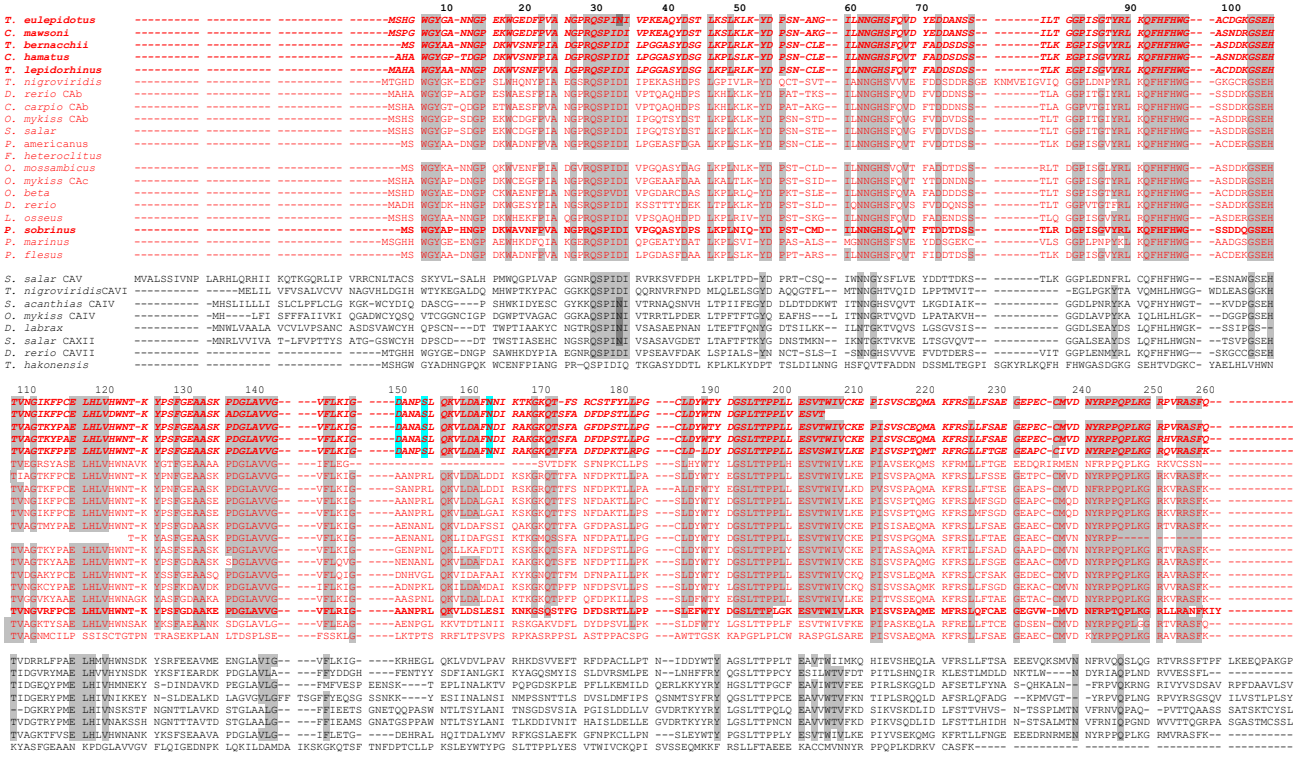

Figure. 1: Fish carbonic anhydrase (CA) sequences. Multiple alignement of aminoacid sequences of carbonic anhydrases deduced from cDNA sequencing of the antarctic nototenoids *Trematomus bernacchii, T. eulepidotus, T. lepidorhinus, Chignodraco mawsoni* (partial sequence) and from the mudskipper *Periophtalmus sobrinus*. These sequences are aligned with the sequences deduced for teleost carbonic anhydrase from GenBank (GenBank entries listed in Table II). The aminoacids conserved in all sequences are shaded in grey; the aminoacid conserved in all antarctic teleost sequences are blu. The numbers of amino acid positions refer to the sequences of CAII. CAII sequences (in red) are separated from the sequences of other isoforms (in black) with the black line. Boxed is the anomalous region of the *Platichthys flesus* sequence.

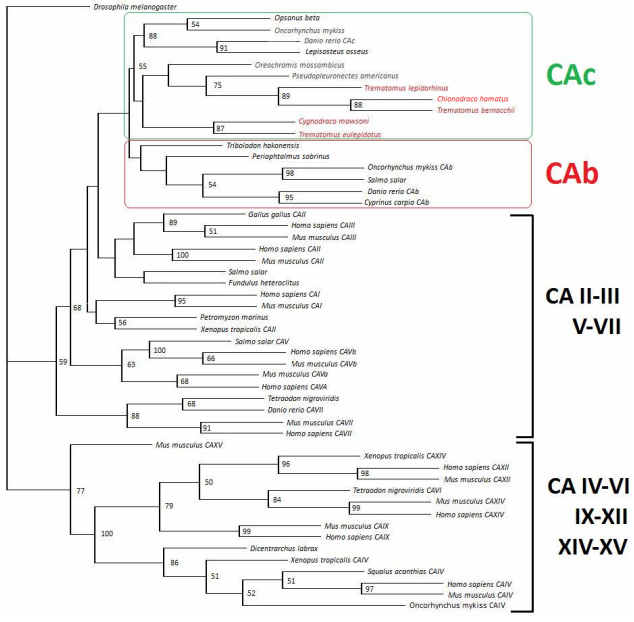

Figure 2: A phylogenetic analysis of the cytoplasmic and membrane-bound carbonic anhydrase isozymes of the α-carbonic anhydrase gene family. The phylogenetic tree was constructed using neighbour joining (reproduced using maximum parsimony method and maximum likelihood method) with support for nodes assessed using bootstrap analysis (1000 pseudoreplicates), and ordered using drosophila CA as an outgroup. Bootstrap values below 50 were not included, denoting poor branch support. Antarctic species are red.

Translated aminoacid sequences are reported in Fig. 1., aligned with CAs from different teleosts (see table II). Within this subset of CA sequences, the identity found for the sequences of antarctic teleosts with the other species is rather high, with percentage of amino acid identities ranging from 74 to 85%.

Several aminoacid signatures characterize the sequence of CAs. In addition to the zinc ligands, attention has been focused on residues that are likely to be included within 10 Å of the zinc atom. These residues are believed to play an important role in defining the catalytic properties of the protein (Marino, Hayakawa et al. 2007). These residues include: the His^{64}, Ser^{65} and Phe^{66}/Leu^{66} important for proton shuttling in all CAIIs where penyalanine is always present in position 66 except in the mudskipper enzyme, where an leucine is present; the hydrophobic residues (positions Trp^{208}, Val^{121}, Leu^{197}, Val^{143}) forming the hydrophobic pocket adjacent to the zinc ion; the residues involved in the hydrogen

bonding network that originates from Zn-hydroxyde complex and involves Gln^{92} with His^{94}, Glu^{117} with His^{119}, and Asn^{243} with His^{96} and to the fourth metal ligand, the exchangeable water molecule Thr^{199}. Table IV shows the alignment of these selections among teleost species. There are only a few aminoacidic sobstitutionswith identical properties. Aromatic idrophobyc Phe^{66} became aliphatic idrophobyc Leu, hydrophobyc Leu^{202} Cys and hydrophobyc Tyr^{244} Phe in *Periophthalmus sobrinus*. In position 116 we found different isofunctional aminoacids: Ala, Phe, Cys and Leu.

The obtained sequences were used to reconstruct the molecular phylogeny of the CA family of proteins together with teleost CA sequences present in database. NJ, ML and MP analyses of vertebrate cytoplasm CAs produced generally well supported phylogenetic trees of similar topology (Fig. 2). These analyses suggested that CAs I, II and III constitute a single monophyletic clade, while the fish cytoplasmic CAs constitute a separate clade. The clade of CA I, II and III of fish and other vertebrates appears after the divergence of CA V and VII.

Different analysis (NJ; MP; ML) revealed that *Periophtalmus sobrinus* is a sister group of paralogs zebrafish CAb-carp CAb and coho salmon-trout CAb. The gar rbc CA was the ancestral sequence in this cluster.

The Antacrtic CA sequences don't group together. The *T. lepidorhinus, T. bernacchii* and *Chionodraco hamatus* group together; *T. lepidorhinus* is sister group. *T. eulepidotus* and *C. mawsoni* cluster together separatelu to the othe Antarctic sequences.

Discussion

The various CA isozymes have a multifunctional role. The most important and well studied concerns the movement of respiratory gases (Lindskog and Silverman, 2000) that involves, especially, CAII and CAIV. For its ubiquitous distribution, this enzyme represents an interesting case to get some insight into the structural signatures relevant for the adaptation of the enzyme to peculiar environments where the organisms live, notably temperature. In the case of heterotherms, it can be expected that the protein is capable to maintain high the catalytic efficiency also at low temperatures (Maffia et al., 2001; Marino et al., 2007).

The CAs sequenced in our study have a coding region of 777 base pairs (259 amino acids, Table III, Fig. 1), and high sequence identity to other known fish CAII sequences (74–85%). The comparison of the amino acid residues located within 10 Å from the active site indicates that the fish cytoplasmic CA are highly similar at the active site level. Furthermore the critical elements for enzyme function are almost entirely conserved throughout teleosts CAII with at most 4 amino acid substitutions (Table IV).

If we compare fish CAII with vertebrate CAII, we can see that there are strongly conservation among amino acidic sequences. The identity among fish CAII and mammalian is 62%, with tetraopod is 60% and with bird is 58%.

This strongly suggests that the catalytic mechanism of CA is highly conserved throughout the vertebrate lineage but much remains to be learned about the way that structural differences in CA influence the specific physiological function of the different isozymes. Furthermore, also the comparisons of fish species adapted to environments with different temperature and salinity (such as the subtidal species *Pseudopleuronectes americanus versus* the intertidal *Periophtalmus sobrinus*, the *Oncorhynchus mykiss* species of temperate waters *versus Trematomus* spp. of Antarctic waters) show that the active site features are highly conserved.

To date, molecular analyses of the phylogeny of the α-CA gene family have been limited by the small number of CA sequence information from non-mammalian vertebrate species (Lund et al., 2002; Tufts et al., 2003). CA V and VII are considered the most ancestral cytoplasmic isozymes (Hewett-Emmett and Tashian, 1991). The mammalian CA I, II and III evolved via a series of gene duplication events (Hewett-Emmett and Tashian, 1991). The fish cytoplasmic CA group emerged prior to the gene duplication event that gave rise to the mammalian CA I, II and III genes, as well as the divergence of other tetraopod vertebrate isozymes, such as *Venous* and chicken CA II (Hewett-Emmett and Tashian, 1991). A further gene duplication event, that occurred at some point in the evolution of teleost fish, or a genome duplication event, that occurred at the origin of modern fish, could account for the presence of two cytoplasmic CAII isozymes in fish: CAb and CAc, where CAb is more expressed in blood (Lin et al., 2008; Esbaugh et al., 2004; Esbaugh et al., 2005) and CAc is more expressed in the gills (Esbaugh et al., 2005; Lin et al., 2008). Generally, the cytoplasmic CA isozyme (CAc) has a lower turnover than the erythrocyte CA isozyme (CAb) (Esbaugh and Tufts, 2006).

In agreement with the above mentioned studies, we found three distinct groups within the α-CA gene family in the tree obtained (Fig. 2). One group contains the cytoplasmic CAs, including mammalian CA I, II, III, V, VII. A further group includes the membrane-bound isozymes: CA IV, IX, XII, XIV, and XV. The last group, well separated from the other vertebrate CAs, is the group of cytoplasmic fish CAs. Our reconstruction also agrees with previous studies since it shows that the division in two different group (CAb and CAc) of fish cytoplasmic CA occurred after the emergence of CA V and VII in vertebrates. Furthermore, the monophyletic origin of fish CAII, suggested by Esbaugh and Tufts (2006) results also from our reconstruction although the bootstrap values are <50 for the dichotomy between fish and other vertebrates (Fig. 2).

The Antarctic continent was subjected, 25–22 million years ago, to a series of tectonic and oceanographic events that created the Antarctic Circumpolar Current (ACC) (Eastman, 2004). This current surrounds the isolated island and creates a natural barrier that forms a unique evolutionary site between the continent and the ACC. Antarctica gradually became isolated and colder and new niches became available to other groups that were diversifying in situ (notothenioids) or immigrating into (liparids and zoarcids). (Anderson, 1999). The natural isolation of this environment provides to become the Antarctic a unique model system to investigate mechanism of adaptative diversification and speciation.

Antarctica can be considered an evolutionary hot spot (Eastman 1993, 2000; Clarke and Johnston 1996). As far as the Antarctic species are concerned, the obtained sequences are typical of CAc (cytoplasmic non-erythrocyte); in contrast, mudskipper CA sequence is typical of CAb (erythrocyte CA). Antarctic CAc does not group closely together: the protein of *T. lepidorhinus* and *T. bernacchii* grouped with the icefish and flounder, while CA of *T. eulepidotus* and *C. mawsoni* grouped within a different cluster. Thus, in our phylogenetic reconstruction, *T. bernacchii* and *C. hamatus,* as well as *C. mawsoni* and *T. eulepidotus* are orthologs. This latter group is paralog to the group including *T. lepidorhinus, T. bernacchii*, *C. hamatus, P. americanus* and *O. mossambicus*. This division suggests a duplication event inside the Antarctic fish, but again the bootstrap value, <50%, demand further analysis.

The phylogenetic relationships of notothenioids support monophyly for both the notothenioids and the Nototheniidae lineages (Ritchie et al., 1997). Phylogenetic analysis of morphology and DNA sequence data (partial mtDNA gene sequences from the control region and cytochrome b (Chen et al., 1998)) show that channichthyids are a monophyletic group, and are one of the most recently derived clades of notothenioids (Balushkin, 2000; Bargelloni et al., 2000; Iwami, 1985). Also the topology of the tree based on Antarctic teleosts 16S rRNA (Verde et al., 2004, Sanchez et al., 2006) supports this phylogenetic relationship.

The tree obtained with our CA sequences is in agreement with the monophyly of notothenioids; however a fine reconstruction within this clade is hampered by the low number of available sequences and the low bootstrap values obtained for the possible dichotomy between Antarctic species.

In this frame, while the sequence comparisons give information on the evolution of the protein, a contribution to understand how the sequence characteristics affect the chemico-physical properties of the various enzymes may derive by the comparison of the three-dimensional reconstruction of the protein obtained through homology modeling using the structure of @@ CA as template. This structural analysis has been performed by one of the coworkers of this paper, S. Marino. This reconstruction allows concluding that the three Antarctic species whose CA sequence has been completely solved (*Trematomus bernacchii, T. lepidorhinus, T. eulepidotus*) *and Periophtalmus sobrinus* obtained through homology modeling were compared to those characterized in a previous paper (Marino et al., 2008).

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

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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 (~35ppt) 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.

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, Johns 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, Taylor 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, Bottcher et al. 1999; Claiborne, Edwards et al. 2002; Marshall 2002; Hirose, Kaneko et al. 2003; Perry, Shahsavarani et al. 2003; Evans, Piermarini 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^- exchange, with HCO_3^- provided by transepithelial transport and/or by CA-mediated hydration of endogenous epithelial CO_2 (Grosell 2006; Grosell, Genz et al. 2009a). Hydration of CO_2 also liberates H^+, which are eliminated mainly across the basolateral membrane of the intestinal epithelium (Grosell 2001; Grosell, Wood et al. 2005; Genz, Taylor 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, Genz et al. 2009a).

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 12h after transfer (Scott, Claiborne 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 1984), while flounder (*Platichthys flesus*) showed no significant differences in CA levels between seawater and freshwater-adapted fish (Mashiter 1975; Sender, Bottcher et al. 1999). Similarly, no relationship to salinity was reported for CA activity in gills of the European eel (*Anguilla anguilla*; (Haswell, Raffin 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, Bastrop et al. 1992).

Considering the intestine, expression or activity of CA increased two- to fourfold 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, Long Ky 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, Taylor 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, Taylor 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 this 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.

Materials and methods

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 ectoparacites (McDonald, Grosell 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 hours prior to sampling. Fish were maintained in the lab and used according to an IACUC-approved University of Miami animal care protocol.

Cloning of toadfish cytoplasmic carbonic anhydrase (CAc)

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.

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), *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.

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

Primer	Sequence (5'- 3')
QFH (F)*	CAGTTYCAYTTCCAYTGGGG
QSPIDI (R)*	CAGTCTCCHATWGAYAT
ReCA (R)*	ACRATCCAGGTGACRCTCTC
Anchor (R)*	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

*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).

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 30s, 72°C for 3 min followed by five cycles of 94°C for 30s, 68°C for 30s and 72°C for 4 min followed by 25 cycles of 94°C for 30s, 68°C for 30s and 72°C for 4 min. Products from all PCR reactions were gel-purified with QIAquick® spin columns (Qiagen), TA-cloned into the pCR® 2.1-TOPO® vector (Invitrogen) and sequenced.

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, 2002) 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).

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 and co-workers at different times following the salinity challenge (Grosell, Genz et al. 2009a) 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 (*C*T) values normalized to EF1α (tissue distribution) using a modification of the delta-delta-*C*T method (Livak and Schmittgen 2001). CT values for *EF1a* did not change following transfer to 60 ppt.

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 2 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) activity was measured at 4 °C employing the ΔpH method as described in (Tufts, Gervais 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, Tufts et al. 1993) at 4 °C to remove cellular debris, mitochondria and membrane fractions. The resulting supernatant was assumed to contain the cytosolic fraction.

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.

Results

Sequencing and Phylogenetic analysis

The cloned cytoplasmic carbonic anhydrase (CAc) transcript from gulf toadfish (GenBank accession number GQ443599) is 1,827 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).

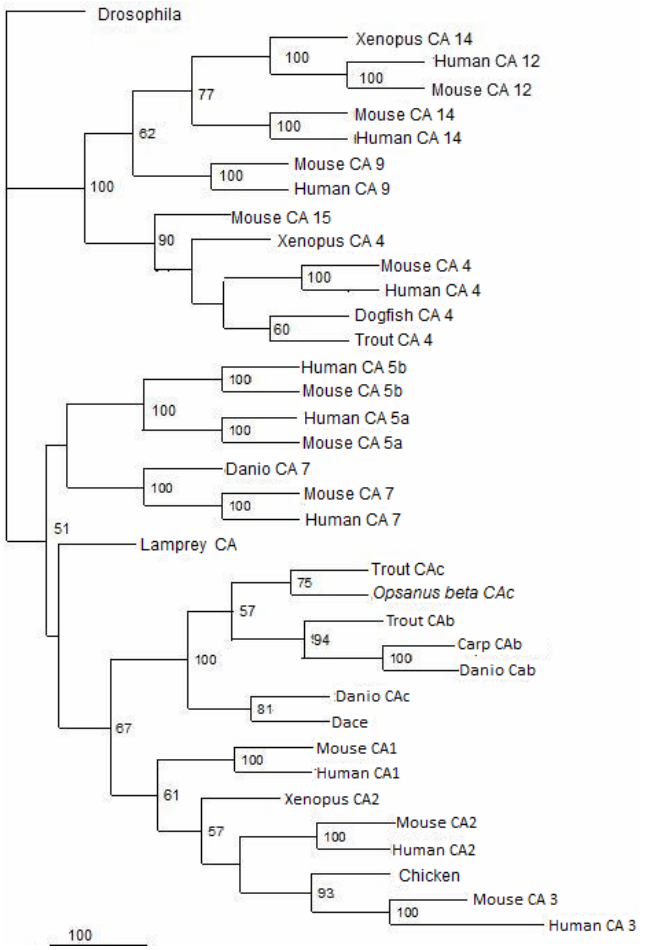

Figure 1: A phylogenetic analysis of the cytoplasmic and membrane-bound CA isozymes of the α-CA gene family. The phylogenetic tree was constructed using neighbour joining with support for nodes assessed using bootstrap analysis (1000 replicates), and ordered using drosophila CA as an outgroup. Bootstrap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

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 hours 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 analysed for CA expression. However, perfused gills were 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 cytoslic activity (Fig. 5).

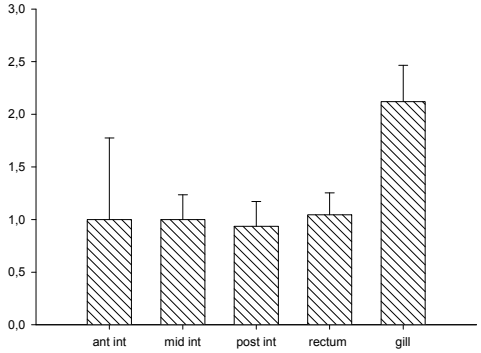

Figure 2: Relative mRNA expression of cytoplasmic carbonic anhydrase in gill, intestine and rectum of seawater acclimated gulf toadfish (Opsanus beta). Values are means ± SEM from n=6 (for gill) and n=8 (for other tissues) different fish.

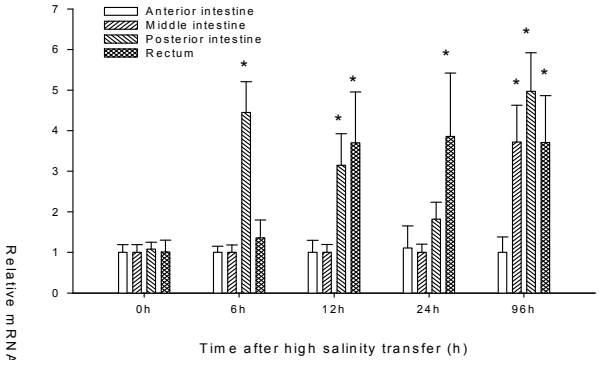

Figure 3: Relative mRNA expression of gulf toadfish (Opsanus beta) cytoplasmic carbonic anhydrase in anterior, middle, posterior intestine and rectum following transfer from

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).

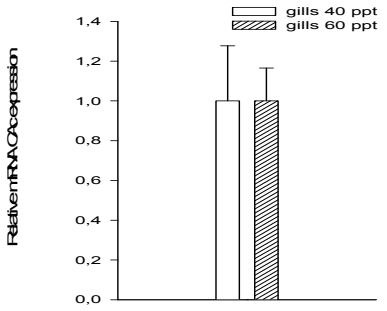

Figure 4: Relative mRNA expression of gulf toadfish (Opsanus beta) cytoplasmic carbonic anhydrase in gill following transfer from normal seawater (40 ppt) to hypersaline water (60 ppt). Values are means ± SEM from n=8 different fish.

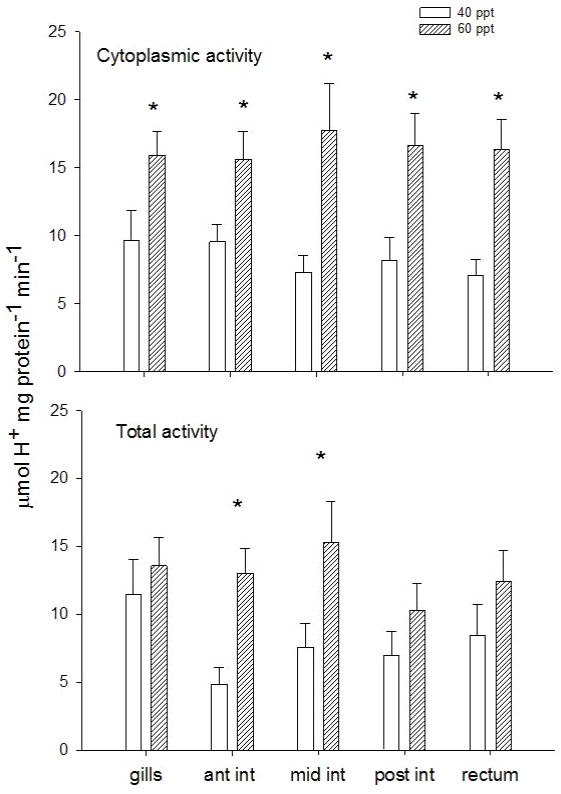

Figure 5: Activity of cytoplasmic (upper panel) and total (lower panel) carbonic anhydrase in gulf toadfish (Opsanus beta) anterior, middle and posterior intestine, rectum and gills. Controls (40 ppt) and two weeks hypersalinity (60 ppt) exposed specimens. Values are means ± SEM from n=10 different fish.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

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, Lund et al. 2004; Esbaugh, Perry et al. 2005; Lin, Liao et al. 2008) and CAc is expressed preferentially in gills (Esbaugh, Perry et al. 2005; Lin, Liao et al. 2008). The phylogenetic tree obtained suggests the cloned toadfish CA gene is the 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, Dyment et al. 2002; Tufts, Esbaugh et al. 2003; Esbaugh, Lund et al. 2004; Esbaugh, Perry et al. 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, Genz et al. 2009a).

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 hyper salinity 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, Bottcher et al. 1999; Henry and Swenson 2000; Esbaugh and Tufts 2004; Esbaugh, Perry 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, rather than branchial CAc is important for survival in salinities above normal seawater.

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 hours 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 day 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.

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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