The Journal of Experimental Biology 212, 3837-3845
Published by The Company of Biologists 2009
doi:10.1242/jeb.034116

Carbonic anhydrase expression and CO_2 excretion during early development in zebrafish *Danio rerio*

K. M. Gilmour*, K. Thomas, A. J. Esbaugh and S. F. Perry

Department of Biology and Centre for Advanced Research in Environmental Genomics, University of Ottawa, Ottawa, ON, Canada

*Author for correspondence (kgilmour@uottawa.ca)

Accepted 27 August 2009

SUMMARY

Carbonic anhydrase (CA) is critical for CO_2 excretion in adult fish, but little is known of the expression or function of CA during early development. The present study examined the hypothesis that, as rates of CO_2 production increased during early development in zebrafish (*Danio rerio*), CA would become necessary for effective CO_2 excretion, and that the pattern of CA expression during early development would reflect this transition. Real-time RT-PCR was used to examine the mRNA expression of the two main intracellular CA isoforms over a time course of early development ranging from 0 to 120 h post fertilization (h.p.f.). The mRNA expression of zCAb was generally higher than that of zCAc, particularly during the earliest stages of development. Rates of CO_2 excretion increased approximately 15-fold from 24 to 48 h.p.f. whereas rates of O_2 uptake increased only 6.7-fold over the same period, indicating a relative stimulation of CO_2 excretion over O_2 uptake. Treatment of 48 h.p.f. larvae with the CA inhibitor acetazolamide resulted in CO_2 excretion rates that were 52% of the value in control larvae, a significant difference that occurred in the absence of any effect on O_2 uptake. Antisense morpholino oligonucleotides were used to selectively knock down one or both of the main intracellular CA isoforms. Subsequent measurement of gas transfer rates at 48 h.p.f. indicated that CA knockdown caused a significant relative inhibition of CO_2 excretion over O_2 uptake, regardless of which cytosolic CA isoform was targeted for knockdown. These results suggest that between 24 h.p.f. and 48 h.p.f., developing zebrafish begin to rely on CA to meet requirements for increased CO_2 excretion.

Key words: carbonic anhydrase, CO_2 excretion, O_2 uptake, respiratory exchange ratio, zebrafish, *Danio rerio*.

INTRODUCTION

Carbonic anhydrase (CA) is the zinc metalloenzyme that catalyzes the reversible reactions of CO_2, i.e. $CO_2+H_2O \leftrightarrow H^+ + HCO_3^-$. Fish, like mammals (reviewed by Chegwidden and Carter, 2000; Hewett-Emmett, 2000; Sly, 2000; Tashian et al., 2000; Purkerson and Schwartz, 2007; Hilvo et al., 2008), possess an abundance of CA isoforms that differ in molecular sequence, kinetic properties and susceptibility to inhibitors, tissue distribution and subcellular localization (reviewed by Henry and Swenson, 2000; Esbaugh and Tufts, 2006; Gilmour and Perry, 2009). In adult fish, several of these isoforms have been implicated in a specific fashion in physiological processes ranging from CO_2 excretion (Henry and Swenson, 2000; Perry and Gilmour, 2006; Esbaugh and Tufts, 2006), through acid–base balance and ionic regulation (Perry and Gilmour, 2006; Gilmour and Perry, 2009), to ureagenesis and metabolism (Henry, 1996). However, much less is known about the expression or function of CA isoforms during early development in fish. The objective of the present study was to investigate CA expression and function during early development in zebrafish (*Danio rerio*), with a particular focus on the main intracellular CA isoforms and their potential contribution to CO_2 excretion.

Work carried out on zebrafish and rainbow trout (*Oncorhynchus mykiss*) suggests that adult teleost fish possess two closely related cytosolic CA isoforms that differ in tissue distribution and kinetic properties (Rahim et al., 1988; Esbaugh et al., 2004; Esbaugh et al., 2005; Lin et al., 2008) (reviewed by Gilmour and Perry, 2009). One, referred to here as the 'b' isoform, is expressed predominantly in the blood and has a higher catalytic efficiency than the second, 'c', isoform, which is more widely distributed, with high expression in the gills, lower expression in the kidney and little or none in red blood cells (RBCs) (Rahim et al., 1988; Esbaugh et al., 2005; Lin et al., 2008). RBC CA plays a key role in CO_2 excretion by catalyzing the hydration of CO_2 to HCO_3^- to load CO_2 into the blood in the tissues, and by catalyzing the reverse process in the gills, where HCO_3^- is dehydrated to CO_2 that then diffuses out of the blood and into the ventilatory water (see Perry, 1986; Tufts and Perry, 1998; Perry and Gilmour, 2006) (reviewed by Esbaugh and Tufts, 2006). When the availability of RBC CA is reduced, through either severe anaemia (Wood et al., 1982; Gilmour and Perry, 1996; Gilmour and MacNeill, 2003) or treatment with a permeant CA inhibitor such as acetazolamide (e.g. Hoffert and Fromm, 1973; Gilmour et al., 2001), CO_2 excretion is impaired as evidenced by increases in the partial pressure of CO_2 (P_{CO2}) in the arterial blood and the appearance of a respiratory acidosis. By contrast, branchial cytosolic CA contributes to ionic regulation and acid–base balance, but not CO_2 excretion. Cytosolic CA in the branchial epithelium catalyzes the hydration of CO_2 to H^+ and HCO_3^- that can then be used as counter-ions for, respectively Na^+ and Cl^- uptake, transport processes that are critical for both ionic regulation and acid–base balance (reviewed by Evans et al., 2005; Perry and Gilmour, 2006; Tresguerres et al., 2006; Hwang and Lee, 2007; Gilmour and Perry, 2009). Correspondingly, inhibition of branchial CA reduces Na^+ and/or Cl^- uptake (Maetz, 1956; Maetz and Garcia-Romeu, 1964; Kerstetter et al., 1970; Payan et al., 1975; Boisen et al., 2003; Chang and Hwang, 2004) as well as branchial net acid excretion (Georgalis et al., 2006).

Zebrafish embryos and larvae express both zCAb and zCAc (Lin et al., 2008), but the relative expression of zCAb *versus* zCAc as a

function of developmental stage has not yet been reported. Using *in situ* hybridization, zCAc mRNA expression was localized to a subtype of ionocytes in the skin and gills that is enriched in vacuolar-type H^+-ATPase (HR cell), whereas zCAb mRNA expression was detected in blood cells (Lin et al., 2008). A role for zCAc in Na^+ uptake and acid excretion has been proposed on the basis of data showing reduced Na^+ uptake and/or acid excretion in larvae treated with the CA inhibitor ethoxzolamide (Esaki et al., 2007) and in embryos and larvae in which zCAc has been knocked down using antisense morpholino oligonucleotides (Lin et al., 2008). The function of zCAb has not yet been investigated. Moreover, several studies have assessed O_2 uptake in embryonic or larval fish (see reviews by Rombough, 1988; Rombough, 2004), but corresponding data for CO_2 excretion do not appear to be available.

With this background in mind, the present study examined the hypothesis that, as rates of CO_2 production increase during early development in zebrafish, CA becomes necessary for effective CO_2 excretion, and that the pattern of CA expression during early development reflects this transition. That is, increased zCAb mRNA expression was expected to precede or coincide with a developmental time point at which CA becomes beneficial for CO_2 excretion, as demonstrated by significant reductions in CO_2 excretion when CA activity, and specifically CAb activity, is reduced by selective gene knockdown. Relative expression of zCAc and zCAb mRNAs was evaluated using real-time RT-PCR. CO_2 excretion was determined relative to O_2 uptake using closed system respirometry. CA activity was reduced by treatment with acetazolamide, and because acetazolamide inhibits all CA activity, the effects of selective knockdown using antisense morpholino oligonucleotides of zCAc and zCAb were also examined.

MATERIALS AND METHODS

Experimental animals

Mature zebrafish (*Danio rerio* Hamilton) obtained from a commercial supplier (Big Al's, Montreal, Canada) were transported to the University of Ottawa Aquatic Care Facility where they were maintained in fibreglass tanks (4 l) held under a constant 10 h:14 h L:D photoperiod and supplied with aerated, dechloraminated city of Ottawa tap water at 28°C (water ion concentrations (in mmol l^{-1}): Na^+ 0.68, Cl^- 0.13, K^+ 0.02, Ca^{2+} 0.23; water pH 7.2–7.7). Newly spawned (within 30 min) eggs were collected from breeding tanks containing equal numbers of male and female fish. Fertilized eggs were incubated in rearing tanks at 28°C until the desired developmental stage. Hatching normally occurred around 48 h post fertilization (h.p.f.), but larvae were not fed; the oldest larvae used in the present study were 120 h.p.f. All procedures for animal use were carried out in accordance with the animal care guidelines of the University of Ottawa and the Canadian Council on Animal Care.

Time course of cytosolic CA expression by real-time RT-PCR

Real-time RT-PCR was used to assess whole body mRNA expression of the two main intracellular CA isoforms as a function of developmental stage for embryos and larvae ranging from 3 h.p.f. to 120 h.p.f. In addition, the relative mRNA expression of these two CA isoforms in RBCs from adult zebrafish was compared. Adult zebrafish were killed by a blow to the head and blood was collected into microcapillary tubes following caudal severance. Blood samples collected from four to eight zebrafish were pooled to form a single sample.

Primers (Table 1) were designed using Genetool software (BioTools Inc., Jupiter, FL, USA) to amplify an ~100 base pair (bp) section of NM_131110 [termed *zCAb* in this study or *zCA2-like b* by Lin et al. (Lin et al., 2008) and of NM_199215 (termed *zCAc* in this study but also known as *zCA 2-like a*) (Lin et al., 2008)]. On the basis of phylogenetic (see Esbaugh and Tufts, 2006; Gilmour and Perry, 2009) and functional (see Lin et al., 2008) analyses, zCAb was expected to be expressed at high levels in RBCs whereas a broader distribution across tissues with little expression in RBCs was expected for zCAc. All primers were selected for optimal amplification at an annealing temperature of 58°C. The specificity of individual primer pairs was confirmed by sequencing of amplicons.

Total RNA was extracted from homogenized samples (<100 mg, representing ~40–60 embryos or larvae, or blood from four to eight adult zebrafish) using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Embryos, larvae or blood samples were homogenized using a 21 gauge needle attached to a 3 ml syringe until the mixture could pass easily through the needle. Following quantification of RNA by spectrophotometry at 260 nm (Spectramax 340 PC, Molecular Devices, Sunnyvale, CA, USA or ND-1000 NanoDrop, ThermoFisher Scientific, Ottawa, ON, Canada), samples were treated according to the manufacturer's instructions with amplification grade DNase I (Invitrogen) to eliminate any genomic DNA contamination. First-strand cDNA synthesis was then performed on 2 µg of DNase-treated total RNA using random primers (200 ng per reaction) and RevertAid H-minus M-MulV reverse transcriptase (Fermentas, Burlington, ON, Canada) according to the manufacturer's instructions. The final cDNA product was diluted with an equal volume of autoclaved water.

Real-time RT-PCR reactions were performed using a SYBR green master mix kit (Stratagene, Agilent Technologies, Cedar Creek, TX, USA) and Mx3000P Real-Time PCR System with associated software (Stratagene). The thermocycler settings were those suggested by the manufacturer, as was the composition of the reaction, with the exception that the reaction volume was scaled to 12.5 µl from 25 µl. Specificity of primer pairs and the occurrence of primer–dimer formation were monitored by creating and

Table 1. Sequences for real-time RT-PCR primers and antisense morpholino oligonucleotides

Label	Sequence
Real-time RT-PCR primers	
zCAb forward primer	5′-GGA TAT GGA CCA GCT GAC GGG CC-3′
zCAb reverse primer	5′-AGG ATG CTC TTG GTG GTG GCT GG-3′
zCAc forward primer	5′-CGA TAA GCA TAA CGG CCC AGA CAA-3′
zCAc reverse primer	5′-CTA TTG GAG ACT GGC GAG AGC CG-3′
18S forward primer	5′-GGC GGC GTT ATT CCC ATG ACC-3′
18S reverse primer	5′-GGT GGT GCC CTT CCG TCA ATT C-3′
Antisense morpholino oligonucleotides	
zCAb	5′-CAA GCG TGG GCC ATG ATT ATA AAT G-3′
zCAc	5′-AGT GGT CAG CCA TTC CGC CAG CTG T-3′
Standard control	5′-CCT CTTA CCT CAG TTA CAA TTT ATA-3′

evaluating a dissociation curve for each reaction. To confirm that products did not arise from amplification of genomic DNA, 'no reverse transcriptase' control templates, generated by omission of reverse transcriptase during cDNA synthesis, were included in every plate. Standard curves were used to assess reaction efficiency. Templates for standard curves were generated by serial dilution (1:5 using RNase/DNase-free molecular grade water; ThermoFisher Scientific) of embryo cDNA. Reactions were then carried out using 2 µl of template for six diluted samples and a linear regression was calculated for the relationship between cycle threshold (Ct) and relative template concentration. Primer pair efficiencies of 85–115% with an R^2 of at least 0.98 were deemed to be acceptable. Relative mRNA expression of the gene of interest against the reference 18S gene (template diluted 1000-fold) was determined according to the modified Δ-ΔCt method of Pfaffl (Pfaffl, 2001). For purposes of comparison across the developmental time course, mRNA expression of both zCAb and zCAc was calculated relative to the expression of zCAc mRNA at 1 h.p.f. Similarly, expression of zCAc mRNA in adult zebrafish RBCs was calculated relative to that of zCAb.

Measurement of aerobic metabolism by respirometry

O_2 consumption and CO_2 excretion were determined for groups of 40–60 zebrafish embryos or larvae using two closed-cell glass micro-respirometry chambers (2.94 ml volume; Loligo Systems, Tjele, Denmark). Micro-respirometry chambers were used in conjunction with a mini magnetic stirring system (Loligo Systems); each chamber contained a magnetic stirrer bar that was separated from the embryos or larvae by a stainless steel mesh. The chambers were connected individually to a supply of aerated, 28°C water and the entire system was submerged in a water bath held at 28°C. Water P_{O_2} was monitored using fibre-optic O_2 electrodes (FOXY AL-300; Ocean Optics, Dunedin, FL, USA) and a PC running Ocean Optics software. Electrodes were calibrated immediately before each experimental run by immersing them first in zero solution (2 g l^{-1} sodium sulphite) and then in air-saturated water until stable readings were recorded. Water total CO_2 concentration was measured at the beginning and end of each experimental run. Water samples (500 µl) were withdrawn from the chamber into gas-tight Hamilton syringes and total CO_2 content was measured in triplicate on 100 µl samples using a Capni-Con 5 (Cameron Instruments, Port Aransas, TX, USA).

An experimental run commenced once stable P_{O_2} readings were obtained following the addition of a group of embryos or larvae to the micro-respirometry chamber. An initial water sample was withdrawn, the chamber was sealed, and P_{O_2} was monitored as oxygen levels declined owing to consumption by the fish. The experimental run was terminated when water P_{O_2} reached 6666 Pa (~20–90 min depending on the age of the embryos/larvae) and the final water sample was withdrawn. O_2 consumption ($\dot{M}_{O_2}$) was calculated from the rate of change of P_{O_2} taking into account the volume of chamber, mass of embryos/larvae and the solubility coefficient of O_2 in water at 28°C (Boutilier et al., 1984). CO_2 excretion ($\dot{M}_{CO_2}$) was calculated from the difference in total CO_2 content between the initial and final water samples, taking into account the duration of the experimental run and the mass of embryos/larvae. To examine CO_2 excretion relative to O_2 uptake, the respiratory exchange ratio (*RE*) was calculated for each experimental run as $\dot{M}_{CO_2}/\dot{M}_{O_2}$.

For purposes of comparison, O_2 uptake and CO_2 excretion were also determined for adult zebrafish. The experimental approach used was essentially the same as that detailed above, but a single fish was used rather than a group, and the micro-respirometry chamber was replaced by a respirometer fashioned from a 10 ml plastic syringe. Water circulation during the period of closed-system respirometry was achieved using a peristaltic pump that passed water through a short tubing loop; the total volume of the respirometer was approximately 7 ml. Fish were acclimated to the respirometer for 1 h before commencing an experimental run.

Experimental treatments

O_2 consumption and CO_2 excretion were determined for groups of zebrafish embryos or larvae at developmental stages ranging from 3 h.p.f. to 120 h.p.f. To determine whether changes in CO_2 excretion at hatching (~48 h.p.f.) were associated with loss of the egg capsule, the egg capsule was manually removed under a light microscope using fine forceps. The egg capsule functions to protect the developing embryo, but forms a barrier to O_2 diffusion from the bulk water to the embryo (Ciuhandu et al., 2007) and might therefore also impede CO_2 diffusion in the opposite direction. Egg capsule removal was carried out at ~22 h.p.f., and O_2 consumption and CO_2 excretion were then measured at 24 h.p.f. or following an additional 24 h of incubation without the egg capsule, at 48 h.p.f. The contribution of CA to CO_2 excretion was then examined at 48 h.p.f. using one of two approaches, widespread inhibition of CA activity using the permeating sulphonamide inhibitor acetazolamide (Az), or selective knockdown of cytosolic CA isoforms using antisense morpholino oligonucleotides (MOs). In either case, whole-body CA activity was assayed to confirm CA inhibition (see below). Larvae were treated with acetazolamide by incubating them for 15 min in a solution of 2×10^{-4} mol l^{-1} Az. Acetazolamide was dissolved in water in which the fish were to incubated by raising the pH to ~11 with NaOH. Water pH was brought back to approximately neutral (pH 7.2) by titration with HCl prior to the addition of larvae. To selectively inhibit the translation of zCAc, zCAb, or both zCAc and zCAb, specific morpholino oligonucleotides were purchased from Gene Tools LLC (Philomath, OR, USA). The morpholinos (Table 1) were designed to be complementary to the translational start site of zCAb (NM_131110) and zCAc (NM_199215) based on the annotated sequences. Morpholinos were prepared in 1× Danieau buffer (58 mmol l^{-1} NaCl, 0.7 mmol l^{-1} KCl, 0.4 mmol l^{-1} $MgSO_4$, 0.6 mmol l^{-1} $Ca(NO_3)_2$ and 5 mmol l^{-1} Hepes, pH 7.6) containing 0.1% Phenol Red as a visible indicator of successful injection, and were injected into zebrafish embryos at the one- to two-cell stage using a microinjector system (IM 300 programmable microinjector; Narishige, East Meadow, NY, USA) with injection needles pulled from filamented 1.0-mm borosilicate glass (Sutter Instrument, Novato, CA, USA). Embryos were injected with either morpholino singly (4 ng embryo^{-1}), or with both morpholinos together (2 ng embryo^{-1} of each morpholino). Control for the effects of morpholino injection itself was achieved by injecting a separate group of embryos with a nonsense morpholino (4 ng embryo^{-1}; standard control morpholino oligonucleotide; Gene Tools LLC). To confirm the specificity and effectiveness of the morpholinos, fusion constructs were made in which the zCAc and zCAb morpholino target sequences were separately introduced upstream of and in frame with the red fluorescent protein dTomato coding sequence (Shaner et al., 2004) (a kind gift from Marc Ekker). Fusion constructs were generated *via* PCR and directionally ligated into the pCS2+ vector (Turner and Weintraub, 1994) (a kind gift from Marc Ekker) using T4 DNA ligase (Fermentas). Capped and poly(A)$^+$ tailed mRNAs were subsequently generated using the SP6 mMESSAGE mMACHINE *in vitro* transcription system (Ambion; Applied Biosystems Canada, Streetsville, ON, Canada), according

to the manufacturer's specifications. Capped mRNAs (100 pg embryo^{-1}) were then injected alone or with each CA isoform morpholino (4 ng embryo^{-1}) at the one- to two-cell stage. The occurrence of dTomato fluorescence was then examined in larvae at 6 days post fertilization (d.p.f.) using a UV assembly attached to a SMZ-1500 microscope (Nikon; Mississauga, ON, Canada) and photographed using a DXM 1200C digital camera (Nikon).

Measurement of carbonic anhydrase activity

The micro-method of Brion et al. (Brion et al., 1988) was used to assay the CA activity present in 48 h.p.f. control larvae and those treated with acetazolamide, as well as the CA activity of 48 h.p.f. larvae that had been micro-injected with morpholino oligonucleotides. Groups (40–60) of larvae were weighed and then homogenized in 20 volumes of distilled water using a motor-driven Teflon homogenizer. As described by Brion et al. (Brion et al., 1988), the reaction vessel for the assay consisted of a 10×75 mm glass tube held in an ice-water bath and fitted with a rubber stopper containing 18 and 20 gauge needles for sample delivery as well as gas entry (CO_2 at 150 ml min^{-1}) and exhaust. An aliquot (500 µl) of water (uncatalyzed rate) or sample was added to the reaction chamber and gassed with CO_2. The reaction was initiated by the addition to the chamber of 500 µl of indicator buffer (20 mmol l^{-1} imidazole, 5 mmol l^{-1} Tris and 0.2 mmol l^{-1} *p*-nitrophenol) and timed manually until the initially yellow solution turned colourless. To prevent the occurrence of foaming in the reaction medium, 1 µl of octanol (Sigma-Aldrich; Oakville, ON, Canada) was added to all reactions. CA activity was calculated from the ratio of the times for colour disappearance measured for water *versus* active sample, corrected for dilution factor and sample mass, as described by Brion et al. (Brion et al., 1988).

Statistical analysis

Data are reported as means ± 1 standard error (s.e.m.). The effects of developmental stage were analyzed statistically using one-way analysis of variance (ANOVA). The statistical significance of treatment effects was assessed by Student's *t*-tests or one-way ANOVA, as appropriate. Where one-way ANOVA indicated that significant differences existed, the Holm–Sidak method was applied for *post-hoc* multiple comparisons. Equivalent non-parametric tests were substituted where assumptions of normality or equal variance were violated. Statistical analyses were carried out using SPSS SigmaStat v3.0 (Systat Software, Chicago, IL, USA) with the fiducial limit of significance set to 0.05.

RESULTS

Real-time RT-PCR evaluation of the relative mRNA expression of zCAb and zCAc revealed that both were detectable as early as 0 h.p.f. and increased markedly during early development (Fig. 1). The mRNA expression of zCAb was generally higher than that of zCAc, particularly during the earliest stages of development (≤48 h.p.f.). Whereas zCAc mRNA expression remained low during the early stages of development, becoming significantly elevated over the 0 h.p.f. value only at 72, 96 and 120 h.p.f., zCAb mRNA expression was distinctly elevated at 8 h and remained relatively high (although not significantly so) thereafter, with the relative mRNA expression at 72 and 96 h.p.f. also being significantly higher than at 0 h.p.f. (Fig. 1). The relative mRNA expression of the two isoforms was also examined in RBCs sampled from mature zebrafish, where zCAb mRNA expression, at 24.3±39.0 (N=8) was found to be significantly higher (paired Student's *t*-test, P=0.008) than that of zCAc, 0.001±0.001 (N=8; relative to zCAb).

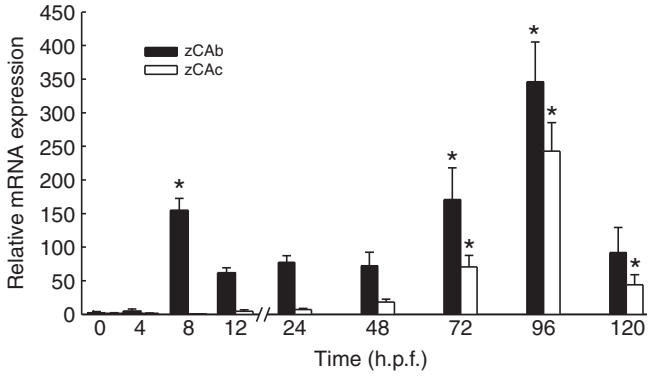

Fig. 1. Relative mRNA expression of the cytosolic carbonic anhydrase isoforms zCAb (NM_131110) and zCAc (NM_199215) in zebrafish (*Danio rerio*) at developmental stages ranging from 1 to 120 h.p.f. All data are expressed relative to mRNA expression of the control 18S gene, and to the 1 h zCAc sample, which was assigned a relative value of 1. Values are means ± 1 s.e.m. (N=8 except at 120 h.p.f., where N=5). An asterisk denotes a significant difference in a CA isoform from the value for that isoform at 0 h.p.f. (one-way ANOVA, P<0.001 for both zCAc and zCAb).

Rates of O_2 consumption and CO_2 excretion were also determined as a function of developmental stage. To provide context, values for adult zebrafish are presented in Fig. 2, but these data were not included in statistical analyses. Both $\dot{M}_{O_2}$ and $\dot{M}_{CO_2}$ were strikingly and significantly (one-way ANOVA, P<0.001 in both cases) higher at 48 to 120 h.p.f. than at the two earlier stages examined, 3 and 24 h.p.f. (Fig. 2A). However, calculation of *RE* ratios uncovered differences in the relative increases in $\dot{M}_{O_2}$ and $\dot{M}_{CO_2}$. In particular, *RE* ratios at 48, 96 and 120 h.p.f. were significantly higher (one-way ANOVA, P<0.001) than those at 3 and 24 h.p.f. (Fig. 2B), a difference that is indicative of a relative increase in CO_2 excretion between 24 and 48 h.p.f. Because hatching occurs over this period, the effect of egg capsule removal on $\dot{M}_{O_2}$, $\dot{M}_{CO_2}$ and *RE* ratio was examined. Egg capsules were removed at 22 h.p.f. and embryos were assessed at 24 or 48 h.p.f., the rationale being that any impact of hatching alone would be eliminated in embryos subjected to egg capsule removal. Embryos from which the egg capsule had been removed exhibited the same pattern as untreated embryos with the *RE* ratio being significantly higher (Student's *t*-test, P=0.014) at 48 h.p.f. (1.15±0.04, N=5) than at 24 h.p.f. (0.82±0.10, N=4), a finding that suggests that the relative increase in CO_2 excretion at 48 h.p.f. is not due to hatching.

Because 48 h.p.f. was the first developmental stage at which higher *RE* ratios were detected, the potential contribution of CA to CO_2 excretion was examined at this time point. Whole-body CA activity in 48 h.p.f. larvae treated with acetazolamide was 38% of the value for untreated larvae (Fig. 3C), a difference that was significant (Student's *t*-test, P=0.002). Acetazolamide-treated larvae exhibited significantly lower $\dot{M}_{CO_2}$ than control larvae in the absence of any impact on $\dot{M}_{O_2}$ (Student's *t*-tests, P=0.001 for $\dot{M}_{CO_2}$ and 0.644 for $\dot{M}_{O_2}$), an effect that resulted in a correspondingly significant difference (Mann–Whitney rank sum test, P=0.011) in *RE* ratio between control and acetazolamide-treated larvae (Fig. 3). Acetazolamide inhibits most or even all CA isoforms (although with differing sensitivity) (see Hilvo et al., 2008), and therefore the effects of selective knock down of zCAb or zCAc were examined using antisense morpholino oligonucleotides. Injection of embryos with one or both CA MOs resulted in significantly lower (one-way ANOVA, P=0.001) whole-body CA activity in 48 h.p.f. larvae than

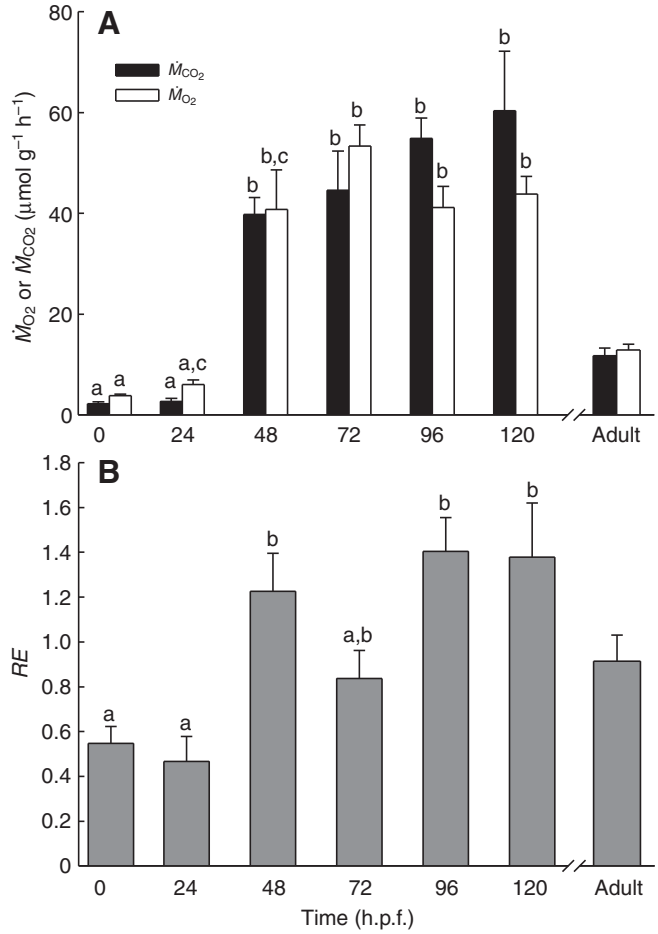

Fig. 2. Effects of developmental stage on (A) O_2 uptake ($\dot{M}_{O_2}$) and CO_2 excretion ($\dot{M}_{CO_2}$), and (B) respiratory exchange ratio (RE=$\dot{M}_{CO_2}$/$\dot{M}_{O_2}$) as measured by closed-system respirometry for zebrafish (*Danio rerio*) embryos and larvae. Values are means ± 1 s.e.m. (N=6–10). Different letters above the bars indicate that the values are significantly different (one-way ANOVA, P<0.001 for $\dot{M}_{O_2}$, $\dot{M}_{CO_2}$ and RE). Data for adult zebrafish are presented for comparison but were not included in the statistical analysis.

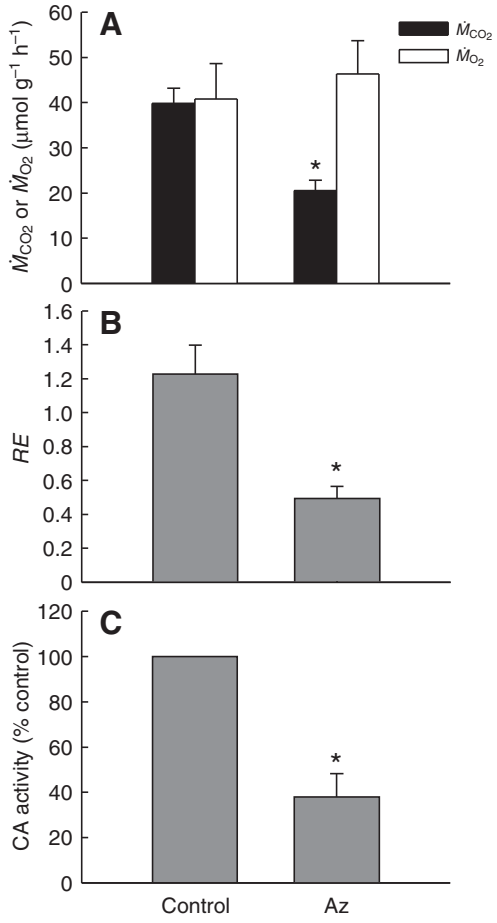

Fig. 3. Effects of acetazolamide (Az) treatment on (A) O_2 uptake ($\dot{M}_{O_2}$) and CO_2 excretion ($\dot{M}_{CO_2}$), (B) respiratory exchange ratio (RE=$\dot{M}_{CO_2}$/$\dot{M}_{O_2}$) and (C) carbonic anhydrase activity of zebrafish (*Danio rerio*) larvae at 48 h.p.f. Values are means ± 1 s.e.m. (N=6–10 for respirometry and 4 for CA assays). An asterisk indicates a significant difference between data for control and acetazolamide-treated larvae (Student's t-test, P=0.011 for $\dot{M}_{CO_2}$, 0.644 for $\dot{M}_{O_2}$, 0.011 for RE and 0.002 for CA activity). Note that CA activities are presented as a percentage of the control value, but statistical analysis was carried out on the actual activity values.

in control larvae at the same stage (Fig. 4C); morphant CA activity ranged from 39% to 69% of the control value. Similarly, RE ratios for zCAb, zCAc and zCAb+zCAc morphants at 48 h.p.f. were significantly lower (one-way ANOVA, P<0.001) than values for control larvae (Fig. 4B). Interestingly, $\dot{M}_{CO_2}$ was unaffected by zCAc and zCAb knockdown (one-way ANOVA on ranks, P=0.551), with the differences in RE ratio reflecting a trend for higher $\dot{M}_{O_2}$ in morphant larvae, a trend that was significant for the double morphant (one-way ANOVA, P=0.01; Fig. 4A). It is also noteworthy that no differences were detected between zCAb and zCAc morphants. Morpholino specificity was confirmed by examination of fluorescence in zebrafish larvae (6 dpf) that were injected as one- or two-cell embryos with an RNA construct, consisting of zCAb or zCAc together with a fluorescent reporter (dTomato), either alone or in conjunction with one of the two morpholinos. Fluorescence was eliminated in larvae that had been co-injected as embryos with both RNA and morpholino for the same CA isoform, but remained in larvae that had been co-injected with RNA for one CA isoform and the morpholino against the other CA isoform (Fig. 5).

DISCUSSION

Rates of O_2 uptake have been measured for early developmental stages in a variety of fish species (e.g. Rombough, 1988), and values measured in the present study (3.8–53.4 μmol g^{-1} h^{-1} for zebrafish of 3–120 h.p.f.; Fig. 2) were comparable with those reported in other studies carried out on zebrafish of similar developmental stage and at similar temperatures (Pelster and Burggren, 1996; Barrionuevo and Burggren, 1999; Bagatto et al., 2001; Grillitsch et al., 2005; Rombough and Drader, 2009). By contrast, the present study appears to be the first to report measurements of CO_2 excretion during early development in a fish. Rates of CO_2 excretion (2.2–60.3 μmol g^{-1} h^{-1} for zebrafish of 3–120 h.p.f.; Fig. 2) were comparable with those of O_2 uptake, but calculation of RE ratios revealed significant differences in the rate of CO_2 excretion relative to O_2 uptake with developmental stage. Under steady state conditions, the RE ratio is expected to equal the respiratory quotient (the ratio of CO_2 production to O_2 consumption at the cellular level) with values between ~0.7 and 1.0 depending on the metabolic substrate (Burggren and Roberts, 1991). As endogenous feeders that

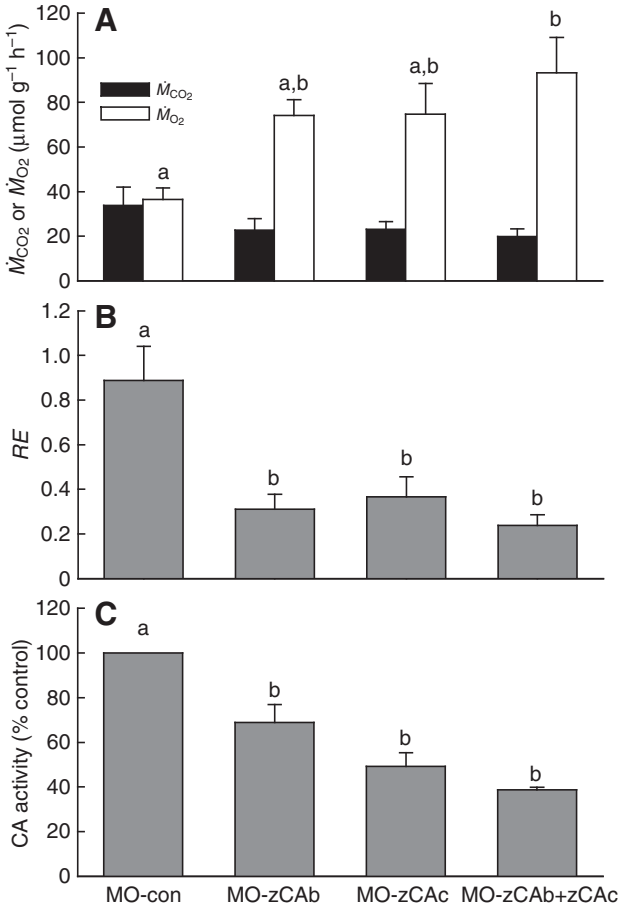

Fig. 4. Effects of CA isoform-specific knockdown on (A) O_2 uptake ($\dot{M}_{O_2}$) and CO_2 excretion ($\dot{M}_{CO_2}$), (B) respiratory exchange ratio (RE=$\dot{M}_{CO_2}$/$\dot{M}_{O_2}$), and (C) carbonic anhydrase activity of zebrafish (*Danio rerio*) larvae at 48 h.p.f. Embryos were injected at the one- to two-cell stage with morpholino oligonucleotides (MOs) designed to inhibit translation of zCAb (MO-zCAb) or zCAc (MO-zCAc). A double morphant was injected with both morpholino oligonucleotides (MO-zCAb+zCAc) and controls were injected with a nonsense morpholino (MO-con). Values are means ± 1 s.e.m. (N=5–7 for respirometry and 2–3 for CA assays). Different letters above the bars indicate that the values are significantly different (one-way ANOVA, P=0.551 for $\dot{M}_{CO_2}$, 0.01 for $\dot{M}_{O_2}$, <0.001 for RE and 0.001 for CA activity). Note that CA activities are presented as a percentage of the control value, but statistical analysis was carried out on actual activity values.

rely on yolk lipids and amino acids (Kamler, 2008), zebrafish embryos and larvae would be expected to exhibit RE ratios or respiratory quotients of 0.7 (lipid) to 0.8 (amino acids). The RE ratio will deviate from the respiratory quotient when CO_2 is stored in the tissues (resulting in RE values <0.7) or when stored CO_2 is eliminated (resulting in RE values >1.0). In the present study, RE ratios prior to hatching were 0.5–0.7, whereas RE ratios for larvae were 1.2–1.4 with the exception of 72 h.p.f. larvae, for which the RE ratio was 0.8. This pattern indicates that CO_2 excretion during embryonic development did not match O_2 uptake, suggesting storage of CO_2 in the tissues, whereas conditions post-hatch favoured CO_2 excretion in excess of O_2 uptake.

Several factors could account for, or contribute to, the relative enhancement of CO_2 excretion post-hatch. Prior to hatching, the developing embryo is surrounded by the egg capsule, a multilayer, acellular, protective shell that forms a barrier to O_2 diffusion from the bulk water to the embryo (Ciuhandu et al., 2007; see also Rombough, 1988) and could also therefore limit CO_2 diffusion away from the embryo. However, embryos subjected to egg capsule removal at 22 h.p.f. and tested at 24 or 48 h.p.f. exhibited the same pattern of enhanced CO_2 excretion at 48 h.p.f. as intact embryos allowed to hatch normally, indicating that the relative suppression of CO_2 excretion prior to hatching was not the result of CO_2 retention by the egg capsule. An alternative possibility is that CO_2 retention during embryonic development reflects a requirement for HCO_3^- for urea synthesis. Zebrafish embryos excrete ~80% of their nitrogenous waste as urea, but switch from ureotely to ammonotely at hatching, a transition that is associated with increases in the expression of ammonia transporters (Rh glycoproteins) (Braun et al., 2009). Thus, ureotelism during early development may be a strategy to prevent ammonia toxicity under conditions where the rate of ammonia excretion is limited by a lack of ammonia transporters (Braun et al., 2009). Urea is formed in embryonic and larval teleost fish from glutamine (as the nitrogen donor) and HCO_3^- *via* the ornithine–urea cycle (e.g. Wright et al., 1995; Chadwick and Wright, 1999; Barimo et al., 2004), and at least in adult toadfish, the HCO_3^- for urea synthesis is supplied by CA-catalyzed hydration of metabolic CO_2 (Walsh et al., 1989). Diversion of CO_2 into urea synthesis during embryonic development would lower CO_2 excretion relative to O_2 uptake. Quantitatively, however, this effect is not large enough to account for the discrepancy in rates of CO_2 excretion *versus* O_2 uptake prior to hatching, since rates of urea excretion at 0–24 h.p.f. are 0.01–0.02 μmol urea g^{-1} h^{-1} (Braun et al., 2009) whereas over the same period O_2 uptake exceeds CO_2 excretion by 1.6–3.4 μmol g^{-1} h^{-1} (Fig. 2). Bone formation might also result in CO_2 storage as carbonate (Pellegrino and Biltz, 1965), although bone formation extends well beyond hatching. It is likely that a number of factors, including urea synthesis and bone formation, contributed to the RE values <0.7 detected prior to hatching in the present study.

RE values >1.0 occurred post-hatching, a time when not only O_2 uptake and CO_2 excretion increased markedly, but also when ammonia excretion exceeds urea excretion (Braun et al., 2009). CO_2 excretion at this time clearly benefited from CA activity as evidenced by the significantly lower rates of CO_2 excretion by larvae in which CA activity was inhibited using acetazolamide. In adult teleost fish, the contribution of CA to CO_2 excretion is tied to the RBC localization of CA activity. RBC CA aids in loading CO_2 into the blood at the tissues by catalyzing the hydration of CO_2 to H^+, which is buffered by haemoglobin, and HCO_3^-, which exits the RBC *via* band 3 anion exchange; at the gill, this process is reversed to unload CO_2 from the blood with RBC CA catalyzing the dehydration reaction (Perry, 1986; Tufts and Perry, 1998). When RBC CA is inhibited by acetazolamide, $\dot{M}_{CO_2}$ falls and a respiratory acidosis (elevated arterial P_{CO_2} in conjunction with lowered arterial pH) develops [e.g. see figure 9 in Gilmour et al. (Gilmour et al., 2001)]. The elevation of arterial P_{CO_2} is expected ultimately to re-establish CO_2 excretion by increasing the partial pressure gradient for CO_2 diffusion from plasma to water. Proof-of-principle experiments using adult zebrafish exposed to waterborne acetazolamide confirmed this pattern; $\dot{M}_{CO_2}$ fell significantly by 25 min of exposure but showed evidence of recovery by 105 min (data not shown). The acetazolamide-induced lowering of CO_2 excretion in zebrafish larvae implies a significant role for CA in CO_2 excretion.

RBC CA expression in trout is primarily as the CAb isoform (Rahim et al., 1988; Esbaugh et al., 2005) and analysis of RBCs from adult fish indicated that the same is true of zebrafish. Examination of whole body mRNA expression profiles for zCAb and zCAc as a function of developmental stage indicated that zCAb

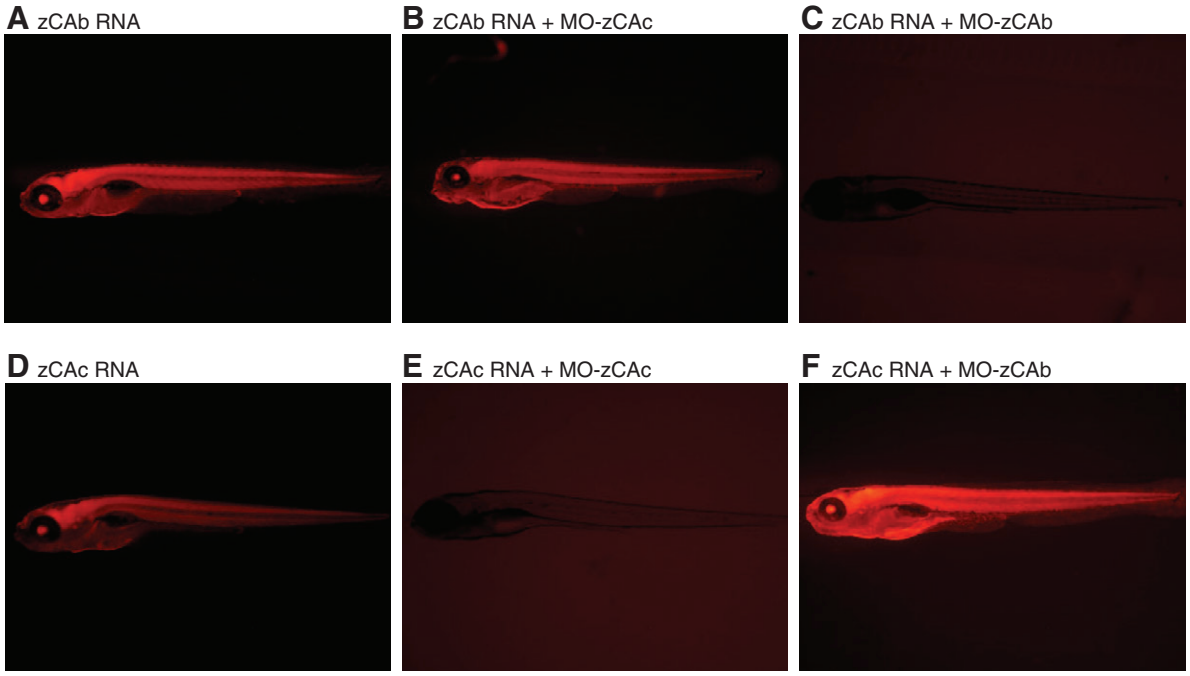

Fig. 5. Images of 6 d.p.f. zebrafish (*Danio rerio*) injected at the one- to two-cell stage with a CA isoform RNA:dTom construct alone or in conjunction with a CA isoform morpholino oligonucleotide. Larvae injected with the construct alone exhibit red fluorescence (A,D) that was abolished when the construct was co-injected with a morpholino against the same CA isoform (C,E) but not when the construct was co-injected with the morpholino against the other CA isoform (B,F).

is the more highly expressed isoform during early development, particularly before 48 h.p.f. Differential tissue distribution of zCAb and zCAc in larval zebrafish was suggested by *in situ* hybridization results, in which zCAc appeared in specific cells of the skin and gill as well as spinal cord neurons and the pronephric duct, whereas zCAb appeared in blood cells of the yolk and tail (Lin et al., 2008). In the present study, the use of a morpholino oligonucleotide targeted to zCAb revealed that zCAb knock down resulted in a significantly lower *RE* ratio, a finding indicative of the inhibition of CO_2 excretion relative to O_2 uptake. Given localization of zCAb to blood cells and by analogy with the model of CO_2 excretion for adult fish, the relative inhibition of CO_2 excretion by zCAb knock down suggests that the RBC is involved in CO_2 excretion at this early stage of development, although convective delivery of CO_2 for elimination is likely to the body surface as a whole and/or the yolk sac surface rather than exclusively the gills (Rombough, 1988; Burggren and Pinder, 1991; Wells and Pinder, 1996; Rombough, 2004). By contrast, several studies using a variety of experimental approaches have demonstrated that O_2 uptake in developing zebrafish is not dependent on haemoglobin O_2 transport (*via* RBCs) until at least 12–14 d.p.f. (Pelster and Burggren, 1996; Weinstein et al., 1996; Jacob et al., 2002; Rombough and Drader, 2009; see review by Rombough, 2004) even though erythrogenesis is initiated around 15 h.p.f. and RBCs containing haemoglobin are present in the circulation by 24 h.p.f. (Weinstein et al., 1996; Brownlie et al., 2003). It is not clear why CO_2 excretion would rely on convective gas transport at a time when O_2 delivery does not. However, the apparent requirement for CA and possibly RBCs for effective CO_2 excretion in larvae may account for the appearance of haemoglobin-containing RBCs long before they are needed for O_2 delivery.

With this scenario, impairment of CO_2 excretion in larval zebrafish would be predicted to occur following selective inhibition of zCAb but not zCAc. Surprisingly, however, the use of a morpholino oligonucleotide targeted to zCAc also resulted in a significantly lower *RE* ratio, i.e. relative inhibition of CO_2 excretion. An explanation for this result is not obvious. The specificity of each morpholino oligonucleotide for the cytosolic CA isoform against which it was designed was confirmed experimentally. In addition, significantly lower levels of CA activity were measured for CA-morphant larvae than for controls, indicating that the morpholino oligonucleotides were effective in lowering CA activity. More specific confirmation of lowered protein levels for each isoform must await development of isoform-specific zCA antibodies. zCAc has been localized to the HR (proton pump-rich) cells in the skin and gills of larvae, where it is postulated to catalyze the hydration of CO_2 to produce protons that are pumped out across the apical membrane as part of the Na^+ uptake mechanism (Hwang and Lee, 2007; Lin et al., 2008). Knock down of zCAc in this location would be expected to inhibit proton excretion, for which there is experimental support (Lin et al., 2008), but might if anything be expected to increase CO_2 excretion by reducing the rate at which molecular CO_2 is converted within the cell to protons and HCO_3^-. It is important to note, however, that knock down of either cytosolic CA isoform is likely to affect the expression profiles of genes associated with CA isoform function, a phenomenon that can lead to confusing results. For example, Lin et al. (Lin et al., 2008) reported increased (rather than reduced) Na^+ uptake by 96 h.p.f. larvae in which zCAc was knocked down, and attributed this apparently contradictory effect at least in part to zCAc knockdown-induced changes in the mRNA expression of several other genes involved in H^+ excretion/Na^+ uptake (a membrane-bound CA isoform, V-type H^+-ATPase, and a Na^+/H^+ exchanger). This type of effect might also account for the interesting observation of elevated $\dot{M}_{O_2}$ in CA morphants. Detailed and quantitative information on the patterns of CA isoform expression in the RBCs of larval zebrafish is also required. Characterization of globin genes in embryonic and larval

zebrafish has revealed complex patterns of globin gene expression and globin switching through development (Brownlie et al., 2003). Whether analogous changes in RBC CA isoform expression occur during zebrafish development is unknown.

In conclusion, the results of the present study implicate CA in CO_2 excretion during early development in zebrafish. Whereas CO_2 excretion in embryos is low relative to O_2 uptake, possibly owing to conversion of CO_2 to HCO_3^- for use in urea synthesis and/or bone formation, hatching is associated with a relative enhancement of CO_2 excretion that is reliant, at least in part, on CA activity. The involvement of CA in CO_2 excretion in larval zebrafish may imply a role for the RBC and could help to explain the appearance of RBCs early in development. In particular, the selective advantage of early haemoglobin synthesis has not been obvious given that it is not needed for O_2 transport (Rombough and Drader, 2009). Based on the findings of the present study, i.e. that CA and possibly RBCs are involved in CO_2 excretion during early development in zebrafish, it is tempting to speculate that haemoglobin synthesis early in development may be driven by the role it plays in buffering H^+ used in CO_2 hydration–dehydration reactions.

This study was supported by Natural Sciences and Engineering Research Council (NSERC) of Canada Discovery and Research Tools & Instruments grants to S.F.P. and K.M.G. Vishal Saxena and Amira Mohamed are thanked for their help with zebrafish embryo injections, without which the study would not have been possible. Luc Poitras and Marc Ekker are thanked for their kind gifts of the dTomato construct and pCS2+ vector. The assistance of Shelby Steele and Tammy Rodela in removing the egg capsule from embryos was appreciated. A.J.E. was the recipient of an NSERC postdoctoral fellowship, while K.T. received awards from the University of Ottawa and the NSERC USRA program.

REFERENCES

Bagatto, B., Pelster, B. and Burggren, W. W. (2001). Growth and metabolism of larval zebrafish: effects of swim training. *J. Exp. Biol.* **204**, 4335-4343.

Barimo, J. F., Steele, S. L., Wright, P. A. and Walsh, P. J. (2004). Dogmas and controversies in the handling of nitrogenous wastes: Ureotely and ammonia tolerance in early life stages of the gulf toadfish, *Opsanus beta*. *J. Exp. Biol.* **207**, 2011-2020.

Barrionuevo, W. R. and Burggren, W. W. (1999). O_2 consumption and heart rate in developing zebrafish (*Danio rerio*): influence of temperature and ambient O_2. *Am. J. Physiol.* **276**, R505-R513.

Boisen, A. M. Z., Amstrup, J., Novak, I. and Grosell, M. (2003). Sodium and chloride transport in soft water and hard water acclimated zebrafish (*Danio rerio*). *Biochim. Biophys. Acta* **1618**, 207-218.

Boutilier, R. G., Heming, T. A. and Iwama, G. K. (1984). Appendix: Physicochemical parameters for use in fish respiratory physiology. In *Fish Physiology* (ed. W. S. Hoar and D. J. Randall), pp. 403-430. London: Academic Press, Inc.

Braun, M. H., Steele, S. L., Ekker, M. and Perry, S. F. (2009). Nitrogen excretion in developing zebrafish (*Danio rerio*): A role for Rh proteins and urea transporters. *Am. J. Physiol.* **296**, F994-F1005.

Brion, L. P., Schwartz, J. H., Zavilowitz, B. J. and Schwartz, G. J. (1988). Micro-method for the measurement of carbonic anhydrase activity in cellular homogenates. *Anal. Biochem.* **175**, 289-297.

Brownlie, A., Hersey, C., Oates, A. C., Paw, B. H., Falick, A. M., Witkowska, H. E., Flint, J., Higgs, D., Jessen, J., Bahary, N., Zhu, H., Lin, S. and Zon, L. (2003). Characterization of embryonic globin genes of the zebrafish. *Dev. Biol.* **255**, 48-61.

Burggren, W. W. and Pinder, A. W. (1991). Ontogeny of cardiovascular and respiratory physiology in lower vertebrates. *Annu. Rev. Physiol.* **53**, 107-135.

Burggren, W. W. and Roberts, J. (1991). Respiration and metabolism. In *Environmental and Metabolic Animal Physiology. Comparative Animal Physiology, 4th ed.* (ed. C. L. Prosser), pp. 353-435. New York: Wiley-Liss.

Chadwick, T. D. and Wright, P. A. (1999). Nitrogen excretion and expression of urea cycle enzymes in the Atlantic cod (*Gadus morhua* L.): A comparison of early life stages with adults. *J. Exp. Biol.* **202**, 2653-2662.

Chang, I.-C. and Hwang, P.-P. (2004). Cl^- uptake mechanism in freshwater-adapted tilapia (*Oreochromis mossambicus*). *Physiol. Biochem. Zool.* **77**, 406-414.

Chegwidden, W. R. and Carter, N. D. (2000). Introduction to the carbonic anhydrases. *EXS* **90**, 13-28.

Ciuhandu, C. S., Wright, P. A., Goldberg, J. I. and Stevens, E. D. (2007). Parameters influencing the dissolved oxygen in the boundary layer of rainbow trout (*Oncorhynchus mykiss*) embryos and larvae. *J. Exp. Biol.* **210**, 1435-1445.

Esaki, M., Hoshijima, K., Kobayashi, S., Fukuda, H., Kawakami, K. and Hirose, S. (2007). Visualization in zebrafish larvae of Na^+ uptake in mitochondria-rich cells whose differentiation is dependent on *foxi3a*. *Am. J. Physiol.* **292**, R470-R480.

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

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

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

Evans, D. H., Piermarini, P. M. and Choe, K. P. (2005). The multifunctional fish gill: dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. *Physiol. Rev.* **85**, 97-177.

Georgalis, T., Perry, S. F. and Gilmour, K. M. (2006). The role of branchial carbonic anhydrase in acid-base regulation in rainbow trout (*Oncorhynchus mykiss*). *J. Exp. Biol.* **209**, 518-530.

Gilmour, K. M. and MacNeill, G. K. (2003). Apparent diffusion limitations on branchial CO_2 transfer are revealed by severe experimental anaemia in brown bullhead (*Ameiurus nebulosus*). *Comp. Biochem. Physiol.* **135A**, 165-175.

Gilmour, K. M. and Perry, S. F. (1996). The effects of experimental anaemia on CO_2 excretion *in vitro* in rainbow trout, *Oncorhynchus mykiss*. *Fish Physiol. Biochem.* **15**, 83-94.

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

Gilmour, K. M., Perry, S. F., Bernier, N. J., Henry, R. P. and Wood, C. M. (2001). Extracellular carbonic anhydrase in dogfish, *Squalus acanthias*: A role in CO_2 excretion. *Physiol. Biochem. Zool.* **74**, 477-492.

Grillitsch, S., Medgyesy, N., Schwerte, T. and Pelster, B. (2005). The influence of environmental PO_2 on hemoglobin oxygen saturation in developing zebrafish *Danio rerio*. *J. Exp. Biol.* **208**, 309-316.

Henry, R. P. (1996). Multiple roles of carbonic anhydrase in cellular transport and metabolism. *Annu. Rev. Physiol.* **58**, 523-538.

Henry, R. P. and Swenson, E. R. (2000). The distribution and physiological significance of carbonic anhydrase in vertebrate gas exchange organs. *Respir. Physiol.* **121**, 1-12.

Hewett-Emmett, D. (2000). Evolution and distribution of the carbonic anhydrase gene families. *EXS* **90**, 29-76.

Hilvo, M., Innocenti, A., Monti, S. M., De Simone, G., Supuran, C. T. and Parkkila, S. (2008). Recent advances in research on the most novel carbonic anhydrases, CA XIII and XV. *Curr. Pharmaceut. Des.* **14**, 672-678.

Hoffert, J. R. and Fromm, P. O. (1973). Effect of acetazolamide on some hematological parameters and ocular oxygen concentration in rainbow trout. *Comp. Biochem. Physiol.* **45A**, 371-378.

Hwang, P.-P. and Lee, T.-H. (2007). New insights into fish ion regulation and mitochondrion-fich cells. *Comp. Biochem. Physiol.* **148A**, 479-497.

Jacob, E., Drexel, M., Schwerte, T. and Pelster, B. (2002). Influence of hypoxia and of hypoxemia on the development of cardiac activity in zebrafish larvae. *Am. J. Physiol.* **283**, R917.

Kamler, E. (2008). Resource allocation in yolk-feeding fish. *Rev. Fish Biol. Fish.* **18**, 143-200.

Kerstetter, T. H., Kirschner, L. B. and Rafuse, D. D. (1970). On the mechanisms of sodium ion transport by the irrigated gills of rainbow trout (*Salmo gairdneri*). *J. Gen. Physiol.* **56**, 342-359.

Lin, T.-Y., Liao, B.-K., Horng, J.-L., Yah, J.-J., Hsiao, C.-D. and Hwang, P.-P. (2008). Carbonic anhydrase 2-like a and 15a are involved in acid-base regulation and Na^+ uptake in zebrafish H^+-ATPase-rich cells. *Am. J. Physiol.* **294**, C1250-C1260.

Maetz, J. (1956). Les échanges de sodium chez le poisson *Carassius auratus* L. Action d'un inhibiteur de l'anydrase carbonique. *J. Physiol., Paris* **48**, 1085-1099.

Maetz, J. and Garcia-Romeu, F. (1964). The mechanism of sodium and chloride uptake by the gills of a fresh-water fish, *Carassius auratus* II. Evidence for NH_4^+/Na^+ and HCO_3^-/Cl^- exchanges. *J. Gen. Physiol.* **47**, 1209-1227.

Payan, P., Matty, A. J. and Maetz, J. (1975). A study of the sodium pump in the perfused head preparation of the trout *Salmo gairdneri* in freshwater. *J. Comp. Physiol.* **104**, 33-48.

Pellegrino, E. D. and Biltz, R. M. (1965). Bone carbonate and the Ca to P molar ratio. *Nature* **219**, 1261-1262.

Pelster, B. and Burggren, W. W. (1996). Disruption of hemoglobin oxygen transport does not impact oxygen-dependent physiological processes in developing embryos of zebra fish (*Danio rerio*). *Circ. Res.* **79**, 358-362.

Perry, S. F. (1986). Carbon dioxide excretion in fishes. *Can. J. Zool.* **64**, 565-572.

Perry, S. F. and Gilmour, K. M. (2006). Acid-base balance and CO_2 excretion in fish: Unanswered questions and emerging models. *Respir. Physiol. Neurobiol.* **154**, 199-215.

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **29**, e45.

Purkerson, J. M. and Schwartz, G. J. (2007). The role of carbonic anhydrases in renal physiology. *Kidney Int.* **71**, 103-115.

Rahim, S. M., Delaunoy, J.-P. and Laurent, P. (1988). Identification and immunocytochemical localization of two different carbonic anhydrase isoenzymes in teleostean fish erythrocytes and gill epithelia. *Histochem.* **89**, 451-459.

Rombough, P. and Drader, H. (2009). Hemoglobin enhances oxygen uptake in larval zebrafish (*Danio rerio*) but only under conditions of extreme hypoxia. *J. Exp. Biol.* **212**, 778-784.

Rombough, P. J. (1988). Respiratory gas exchange, aerobic metabolism, and effects of hypoxia during early life. In *Fish Physiology* (ed. W. S. Hoar and D. J. Randall), pp. 59-161. London: Academic Press, Inc.

Rombough, P. J. (2004). Gas exchange, ionoregulation, and the functional development of the teleost gill. *Am. Fish. Soc. Symp.* **40**, 47-83.

Shaner, N. C., Campbell, R. E., Steinbach, P. A., Giepmans, B. N. G., Palmer, A. E. and Tsien, R. Y. (2004). Improved monomeric red, orange and yellow fluorescent proteins derived from *Discosoma* sp. red fluorescent protein. *Nature Biotech.* **22**, 1567-1572.

Sly, W. S. (2000). The membrane carbonic anhydrases: from CO_2 transport to tumor markers. *EXS* **90**, 95-104.
Tashian, R. E., Hewett-Emmett, D., Carter, N. and Bergenhem, N. C. H. (2000). Carbonic anhydrase (CA)-related proteins (CA-RPs), and transmembrane proteins with CA or CA-RP domains. In *The Carbonic Anhydrases. New Horizons* (ed. W. R. Chegwidden, N. Carter and Y. H. Edwards), pp. 105-120. Basel, Switzerland: Birkhäuser Verlag.
Tresguerres, M., Katoh, F., Orr, E., Parks, S. K. and Goss, G. G. (2006). Chloride uptake and base secretion in freshwater fish: a transepithelial ion-transport metabolon? *Physiol. Biochem. Zool.* **79**, 981-996.
Tufts, B. L. and Perry, S. F. (1998). Carbon dioxide transport and excretion. In *Fish Respiration* (ed. S. F. Perry and B. L. Tufts), pp. 229-281. San Diego: Academic Press.
Turner, D. L. and Weintraub, H. (1994). Expression of achaete-scute homolog 3 in *Xenopus* embryos converts ectodermal cells to a neural fate. *Genes Dev.* **8**, 1434-1447.
Walsh, P. J., Parent, J. J. and Henry, R. P. (1989). Carbonic anhydrase supplies bicarbonate for urea synthesis in toadfish (*Opsanus beta*) hepatocytes. *Physiol. Zool.* **62**, 1257-1272.
Weinstein, B. M., Schier, A. F., Abdelilah, S., Malicki, J., Solnica-Krezel, L., Stemple, D. L., Stainier, D. Y. R., Zwarkruis, F., Driever, W. and Fishman, M. C. (1996). Hematopoietic mutations in the zebrafish. *Development* **123**, 303-309.
Wells, P. R. and Pinder, A. W. (1996). The respiratory development of Atlantic salmon II. Partitioning of oxygen uptake among gills, yolk sac and body surfaces. *J. Exp. Biol.* **199**, 2737-2744.
Wood, C. M., McDonald, D. G. and McMahon, B. R. (1982). The influence of experimental anaemia on blood acid-base regulation *in vivo* and *in vitro* in the starry flounder (*Platichthys stellatus*) and the rainbow trout (*Salmo gairdneri*). *J. Exp. Biol.* **96**, 221-237.
Wright, P. A., Felskie, A. and Anderson, P. M. (1995). Induction of ornithine-urea cycle enzymes and nitrogen metabolism and excretion in rainbow trout (*Oncorhynchus mykiss*) during early life stages. *J. Exp. Biol.* **198**, 127-135.