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DOES GILL BOUNDARY LAYER CARBONIC ANHYDRASE CONTRIBUTE TO CARBON DIOXIDE EXCRETION: A COMPARISON BETWEEN DOGFISH (*SQUALUS ACANTHIAS*) AND RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

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Summary

***In vivo* experiments were conducted on spiny dogfish (*Squalus acanthias*) and rainbow trout (*Oncorhynchus mykiss*) in sea water to determine the potential role of externally oriented or gill boundary layer carbonic anhydrase in carbon dioxide excretion. This was accomplished by assessing pH changes in expired water using a stopped-flow apparatus. In dogfish, expired water was in acid–base disequilibrium as indicated by a pronounced acidification (ΔpH=–0.11±0.01; *N*=22; mean ± S.E.M.) during the period of stopped flow; inspired water, however, was in acid–base equilibrium (ΔpH=–0.002±0.01; *N*=22). The acid–base disequilibrium in expired water was abolished (ΔpH=–0.005±0.01; *N*=6) by the addition of bovine carbonic anhydrase (5 mg l^{-1}) to the external medium. Addition of the carbonic anhydrase inhibitor acetazolamide (1 mmol l^{-1}) to the water significantly reduced the magnitude of the pH disequilibrium (from –0.133±0.03 to –0.063±0.02; *N*=4). However, after correcting for the increased buffering capacity of the water caused by acetazolamide, the acid–base disequilibrium during stopped flow was unaffected by this treatment (control $\Delta[H^+]$=99.8±22.8 μmol l^{-1}; acetazolamide $\Delta[H^+]$=81.3±21.5 μmol l^{-1}). In rainbow trout, expired water displayed an acid–base disequilibrium (ΔpH=0.09±0.01; *N*=6) that also was abolished by the application of external carbonic anhydrase (ΔpH=0.02±0.01).**

The origin of the expired water acid–base disequilibrium was investigated further in dogfish. Intravascular injection of acetazolamide (40 mg kg^{-1}) to inhibit internal carbonic anhydrase activity non-specifically and thus CO_2 excretion significantly diminished the extent of the expired water disequilibrium pH after 30 min (from –0.123±0.01 to –0.065±0.01; *N*=6). Selective inhibition of extracellular carbonic anhydrase activity using a low intravascular dose (1.3 mg kg^{-1}) of the inhibitor benzolamide caused a significant reduction in the acid–base disequilibrium after 5 min (from –0.11±0.01 to –0.07±0.01; *N*=14). These results demonstrate that the expired water acid–base disequilibrium originates, at least in part, from excretory CO_2 and that extracellular carbonic anhydrase in dogfish may have a significant role in carbon dioxide excretion. However, externally oriented carbonic anhydrase (if present in dogfish) plays no role in catalysing the hydration of the excretory CO_2 in water flowing over the gills and thus is unlikely to facilitate CO_2 excretion.

Key words: dogfish, *Squalus acanthias*, rainbow trout, *Oncorhynchus mykiss*, CO_2 excretion, carbonic anhydrase, benzolamide, acetazolamide, gill, boundary layer.

Introduction

It has been proposed that branchial CO_2 excretion in fish is facilitated by externally oriented carbonic anhydrase either associated with the apical membrane of gill epithelial cells or trapped within overlying mucus (for reviews, see Perry, 1986; Perry and Laurent, 1990; Perry and McDonald, 1993; Swenson, 1990; Gilmour, 1997; Tufts and Perry, 1998). According to this model, the transbranchial diffusion of CO_2 is aided by its catalysed chemical hydration to HCO_3^- and H^+ in the boundary layer adjacent to the gill epithelium (Randall and Wright, 1989; Playle and Wood, 1989; Randall et al., 1991). Thus, despite the likelihood of inadequate convection of boundary layer water to physically remove CO_2, adequate blood-to-water P_{CO_2} diffusion gradients are nevertheless maintained as a result of the instantaneous hydration of molecular CO_2 in the presence of external carbonic anhydrase.

There is morphological evidence for externally oriented carbonic anhydrase in fish. Using immunocytochemistry, Rahim et al. (1988) demonstrated an extensive localisation of carbonic anhydrase to the apical surface of lamellar pavement cells in rainbow trout *Oncorhynchus mykiss*. Owing to its

close association with apical villi and microplicae, Rahim et al. (1988) suggested that carbonic anhydrase is in contact with the external environment. Mucous cells also contain carbonic anhydrase (Rahim et al., 1988), and thus the mucous coat covering the gill epithelium is likely to be an additional site of external carbonic anhydrase, as suggested previously for trout skin mucus (Wright et al., 1986). In dogfish (*Squalus acanthias*), an apical distribution of carbonic anhydrase in gill epithelial cells has also been demonstrated (J. Wilson, personal communication). Surprisingly, however, there is scant physiological evidence to support a role for external carbonic anhydrase in CO_2 excretion. Indeed, the few studies that have been conducted (Wright et al., 1986; Heming, 1986; Lin and Randall, 1990) have produced conflicting results. Using a stopped-flow technique, Wright et al. (1986) reported that the expired water of rainbow trout was in acid–base equilibrium and therefore concluded that external carbonic anhydrase must be available to catalyse the hydration reaction of excretory CO_2; this result was later confirmed (Lin and Randall, 1990). In contrast, however, Heming (1986) demonstrated an acid–base disequilibrium in the expired water of rainbow trout using a modified stopped-flow technique, thereby indicating inadequate carbonic anhydrase catalysis to achieve pH equilibrium. Because of these discrepancies, the current model for CO_2 excretion in fish (see above) is not universally accepted (see Henry and Heming, 1998).

Given the controversy surrounding the potential role of external carbonic anhydrase in CO_2 excretion in fish, the goal of the present study was to assess in detail its possible involvement in an elasmobranch (*Squalus acanthias*) and to re-assess its possible function in rainbow trout (*Oncorhynchus mykiss*). The basic experimental approach was to use a stopped-flow apparatus to determine whether $CO_2/HCO_3^-/H^+$ reactions are in equilibrium or disequilibrium in expired water that has contacted the surface of the gill epithelium, the presumed site of external carbonic anhydrase.

Materials and methods

Experimental animals

Pacific spiny dogfish (*Squalus acanthias*) were collected by angling or by net during trawls by local fishermen and transported to holding facilities at Bamfield Marine Station (BMS; Bamfield, Vancouver Island, British Columbia). They were held for up to 4 weeks in a 75 000 l circular tank provided with flowing sea water at 11 °C. The dogfish were maintained under a 12 h:12 h L:D photoperiod and were fed with herring twice weekly. In the present study, 30 dogfish (mean mass 1367±99 g; mean ± S.E.M.) were used within 4 weeks of their capture.

Freshwater rainbow trout (*Oncorhynchus mykiss*) were obtained from a local hatchery and transported to BMS, where they were acclimated gradually (over 4 weeks) to full-strength sea water (at 11 °C) in outdoor holding tanks. They were fed daily with a commercial trout diet. In the present study, six trout (mean mass 508±46 g) were used within 4 weeks following their acclimation.

Surgical procedures

Fish were anaesthetised in a seawater solution of ethyl-*m*-aminobenzoate (0.1 g l^{-1}; MS-222; Syndel) and transferred to an operating table where the gills were irrigated continuously with the same anaesthetic solution. Either the caudal artery or vein was cannulated using polyethylene tubing (Clay Adams PE 50) filled with heparinised (100 units ml^{-1} sodium heparin) dogfish saline (500 mmol l^{-1} NaCl) or teleost Cortland saline (Wolf, 1963) modified for seawater-adapted trout (160 mmol l^{-1} NaCl). Briefly, a lateral incision was made at the level of the caudal peduncle to expose the haemal arch. The caudal artery or vein was cannulated percutaneously using PE 50 tubing. The wound was closed, and the cannula was secured to the body wall using silk ligatures.

In both species, a cannula (Clay Adams PE 160) was inserted into the buccal cavity to permit sampling of inspired water. In dogfish, expired water was sampled from cannulae (PE 160) anchored directly behind the second gill slit on both sides of the head. In trout, expired water was collected using two different techniques. On one side of the head, a cannula (PE 160) with a heat-flared end was inserted into the opercular cavity through a small hole formed by an 18 gauge needle; the cannula was secured with silk thread. On the other side, expired water was collected by securing a cannula to the body wall so that its tip penetrated approximately 2 mm into the opercular cavity (Wright et al., 1986). The effectiveness of these techniques for sampling expired water was assessed by measuring inspired (P_{IO_2}) and expired (P_{EO_2}) water P_{O_2}. The cannula yielding the lower P_{EO_2} value was used in the experiments. After surgery, fish were placed into individual wooden or Perspex boxes provided with aerated full-strength sea water at ambient temperature. Fish were left to recover for approximately 24 h prior to experimentation.

Experimental protocol

The basic experimental approach was to monitor pH changes in the inspired or expired water using a stopped-flow technique (Wright et al., 1986; Heming, 1986). Specifically, water was drawn *via* a peristaltic pump through a thermostatted chamber (volume 0.1 ml) housing a pH electrode. Flow was maintained at 20 ml min^{-1}, and the lengths of the cannulae were kept constant (80 cm). Thus, the estimated transit time of the expired/inspired water to the pH electrode was 2.4 s. After recording stable inspired water pH for approximately 5 min, the peristaltic pump was turned off, and the pH was monitored for a further 4–6 min. Upon recommencing flow and re-establishing baseline values, the same procedure was repeated for expired water after selecting the more suitable of the two cannulae (see above). Water P_{O_2} measurements were carried out independently of the pH measurements by passing inspired or expired water by siphon through a thermostatted chamber housing a P_{O_2} electrode.

Several experimental series were conducted on dogfish. In a

first series using large dogfish (mean mass 1648±67 g; N=22), the pH changes during stopped flow were assessed in detail. In 12 of these fish, benzolamide (courtesy of Dr Ray Henry) was injected into the circulation (1.3 mg kg^{-1}; 0.4 ml kg^{-1}) after the initial set of stopped-flow measurements to selectively inhibit extracellular carbonic anhydrase activity (Swenson and Maren, 1987; Gilmour et al., 1997). A stock solution of benzolamide (3.25 mg ml^{-1}) was prepared in alkaline dogfish saline (pH≈10) and then lowered to pH 8.5 with HCl. After 5–10 min, the stopped-flow experiment was repeated on expired water. All other experiments were performed on smaller dogfish (mean mass 595±33 g; N=8) to reduce the volume of water in the holding boxes and thus to minimise the quantities of carbonic anhydrase used (see below). Immediately prior to beginning the experiments, water flow to the box was stopped, and the volume of water in the box was reduced to 10 l; the water was aerated vigorously to provide mixing and oxygenation. After an initial set of inspired and expired stopped-flow runs, 50 mg of carbonic anhydrase was added to the water to achieve a final concentration of 5 mg l^{-1}. After 10 min, the inspired and expired stopped-flow measurements were repeated. The box was then flushed with sea water for 10 min to remove the added carbonic anhydrase. Removal of the carbonic anhydrase was confirmed by repeating an expired stopped-flow experiment. Water flow to the box was again stopped, and acetazolamide was added to the water to yield a final concentration of 1 mmol l^{-1}; inspired and expired stopped-flow runs were conducted after 10 min. After flushing the box for 10 min, the fish was injected with acetazolamide (40 mg kg^{-1}) *via* the caudal cannula at 0.5 ml kg^{-1}. The stock solution of acetazolamide (80 mg ml^{-1}) was prepared in alkaline dogfish saline (pH≈10.0) and then adjusted to a final pH of approximately 8.5 using HCl. A final set of inspired and expired stopped-flow runs was performed after 30 min to ensure complete inhibition of red cell carbonic anhydrase (Henry et al., 1988).

Because acetazolamide is a strong buffer and is therefore likely to reduce the magnitude of any expired water pH disequilibrium (independently of carbonic anhydrase inhibition), it was necessary to determine the buffering capacities of normal sea water and of sea water containing 1 mmol l^{-1} acetazolamide. Non-bicarbonate buffering capacity was determined by measuring pH and total CO_2 concentration for seawater samples that had been equilibrated with a range of CO_2 tensions (0–3 mmHg; 1 mmHg=0.133 kPa).

Analytical techniques

Water pH was measured using a Metrohm combination glass pH electrode (model 6.0204.100) connected to a Cole Parmer Chemcadet pH meter. Water P_{O_2} was measured using a Radiometer O_2 electrode housed in a thermostatted cuvette and connected to a Radiometer PHM 73 acid base analyser. Analog outputs from the two meters were converted to digital data using a data-acquisition system (BioPac Systems; Goleta, CA, USA) and software (Acknowledge 3.01).

To determine buffer capacity, 3 ml of sea water was equilibrated for 20 min with humidified gas using a Cameron model DEQ1 dual equilibrator; the temperature of the water was maintained at 13.4 °C. A Cameron Instruments three-channel flowmeter connected in series with a Wösthoff gas-mixing pump (M301 a/f) provided the gases (CO_2 in air). Total CO_2 measurements were performed in triplicate on 50 µl samples using a Corning (model 905) CO_2 analyser. Determinations of pH were performed in triplicate using a Radiometer pH and calomel microelectrode assembly and meter (PHM 72). Seawater [HCO_3^-] was calculated from the Henderson–Hasselbalch equation using constants from Boutilier et al. (1984).

Statistical analysis

Data are presented as mean values ±1 standard error of the mean (S.E.M.). The data were analysed statistically using two-tailed paired Student's t-tests. When parametric test assumptions were violated, the data were analysed by Wilcoxon signed-rank test. All statistical tests, including determinations of normality and variance, were performed using commercial software (Sigmastat 2.03). The fiducial limit of significance was set at 5 % (P<0.05).

Results

Dogfish

Representative original recordings for inspired and expired water stopped-flow experiments are presented in Fig. 1. Prior to stopping the flow, inspired water pH (pHi) was significantly greater than expired water pH (pHe; see also Table 1) and $P_{E O_2}$ was approximately 37 mmHg (4.9 kPa) lower than $P_{I O_2}$ (Table 1). Upon stopping the flow of water through the measuring chamber, pHi remained constant, whereas pHe decreased rapidly and then stabilised at an equilibrium value that was 0.11±0.01 (N=22) lower than that of the flowing water. Upon recommencing water flow, pHe increased rapidly to its initial value. The addition of bovine carbonic anhydrase to the

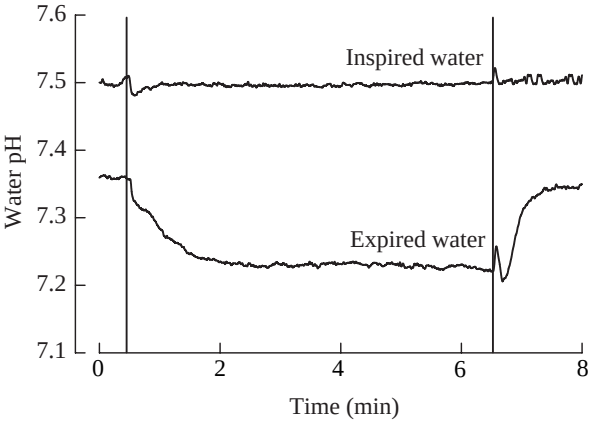

Fig. 1. Representative original recordings of pH changes in expired and inspired water for dogfish (*Squalus acanthias*) during a 6 min period of stopped flow. The stopped-flow interval is bracketed by the two vertical solid lines.

Table 1. *pH and P_{O_2} values for inspired and expired water in dogfish (*Squalus acanthias*) and rainbow trout (*Oncorhynchus mykiss*) as measured under flowing conditions*

	Water pH		Water P_{O_2} (mmHg)	
	Inspired water	Expired water	Inspired water	Expired water
Dogfish (N=24)	7.76±0.04	7.65±0.03*	143.7±3.9	107.0±4.6*
Rainbow trout (N=6)	7.51±0.02	7.44±0.02*	145.0±3.0	107.8±4.6*

Values are shown as means ± 1 S.E.M.

* indicates a statistically significant difference (P<0.05) from the corresponding value in the inspired water.

1 mmHg=0.133 kPa.

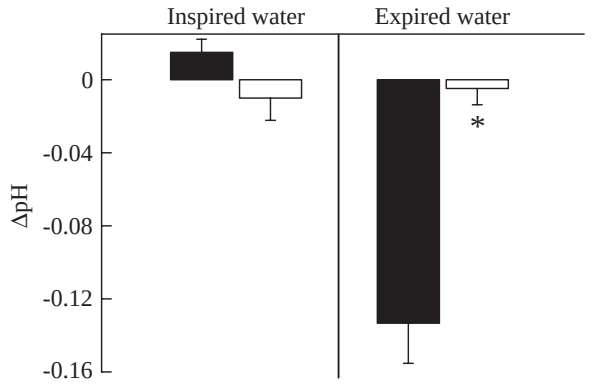

Fig. 2. Changes in pH (ΔpH) in expired and inspired water during 6 min stopped-flow periods in dogfish (*Squalus acanthias*) in the absence (filled columns) or presence (open columns) of external bovine carbonic anhydrase (5 mg l^{-1}). Values are shown as means ± 1 S.E.M.; N=6. An asterisk indicates a statistically significant difference from the control (no carbonic anhydrase) value (P<0.05).

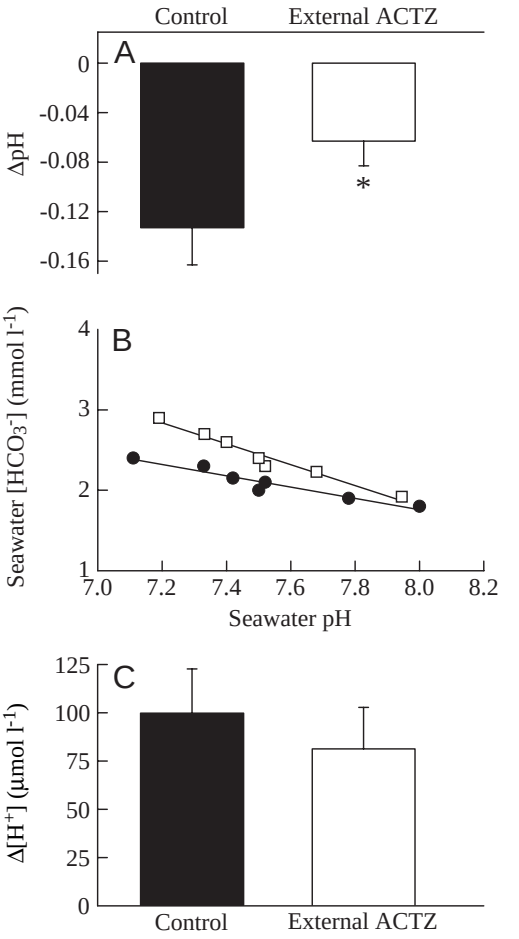

Fig. 3. The effects of external acetazolamide (ACTZ; 1 mmol l^{-1}) on (A) changes in expired water pH (ΔpH) during a 6 min stopped-flow period, (B) seawater non-bicarbonate buffering capacity and (C) proton production during stopped flow (Δ[H^+]) in dogfish (*Squalus acanthias*). The filled columns or symbols represent control sea water whereas the open columns or symbols represent sea water containing acetazolamide (N=4). In B, linear regressions were drawn through the data points: [HCO_3^-]=–0.70pH+7.374; r^2=0.93, P<0.05 for control sea water and [HCO_3^-]=–1.30pH+12.197; r^2=0.96, P<0.05 for acetazolamide-containing sea water. All other details are as in Fig. 2.

external medium abolished the acidification of the expired water during stopped flow without affecting absolute pH or pH changes in the inspired water (Fig. 2). The addition of acetazolamide to the external water significantly reduced the magnitude of the pH change in expired water during stopped flow by approximately 50 % (Fig. 3A). However, the buffering capacity of the sea water was nearly doubled in the presence of acetazolamide (Fig. 3B) from –0.70 to –1.30 mmol l^{-1} pH $unit^{-1}$. Thus, upon factoring in the differences in buffering capacities, the extent of H^+ production during the period of stopped flow was unaltered by external acetazolamide treatment (Fig. 3C). Note that this procedure does not correct for any differences in uncatalysed reaction velocities between the differently buffered media. However, considering that less than 10 % of the uncatalysed reaction is occurring during transit to the pH electrode, such differences in reaction velocities are not likely significantly to influence the results during stopped flow.

Injecting the fish with acetazolamide, a treatment known to inhibit CO_2 excretion, significantly reduced (by 47 %) the extent of the expired water acidification during stopped flow (Fig. 4). Similarly, injection of fish with a low dose of benzolamide to selectively inhibit extracellular carbonic anhydrase also reduced the magnitude of the expired water pH disequilibrium during stopped flow by approximately 34 % (Fig. 5).

Rainbow trout

The expired water of rainbow trout also exhibited an acid–base disequilibrium during stopped flow (ΔpH=–0.09±0.01) that was not present in the inspired water (ΔpH=–0.02±0.01; Fig. 6). Addition of carbonic anhydrase to the external medium eliminated the disequilibrium in the expired water without influencing the inspired water (Fig. 6).

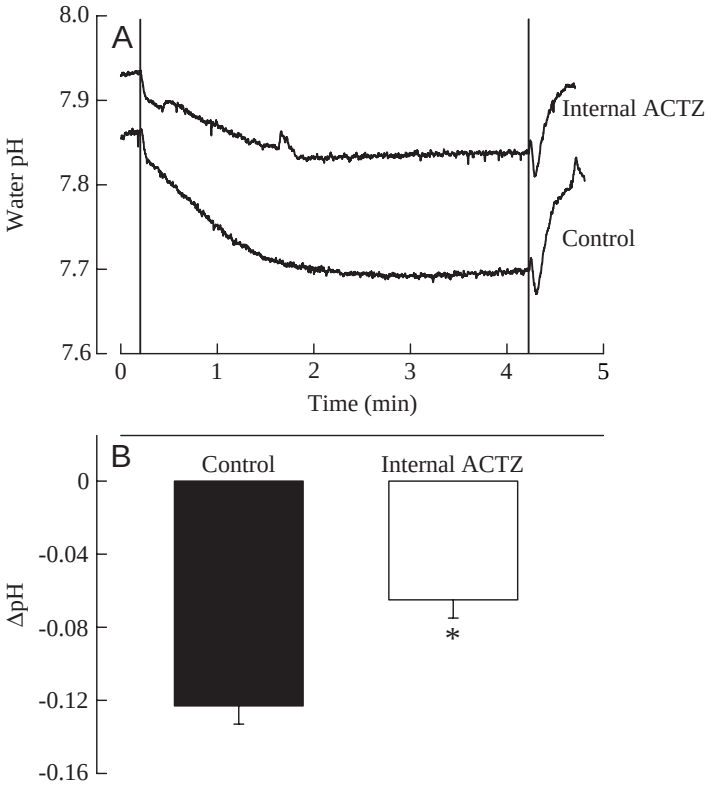

Fig. 4. The effects of internal acetazolamide (ACTZ; 40 mg kg^{-1}) on (A) representative recordings of expired water pH during a 4 min period of stopped flow (bracketed by the vertical line) in dogfish (*Squalus acanthias*) and (B) the mean changes (N=6) in expired water pH (ΔpH) during the stopped-flow period. All other details are as in Fig. 2.

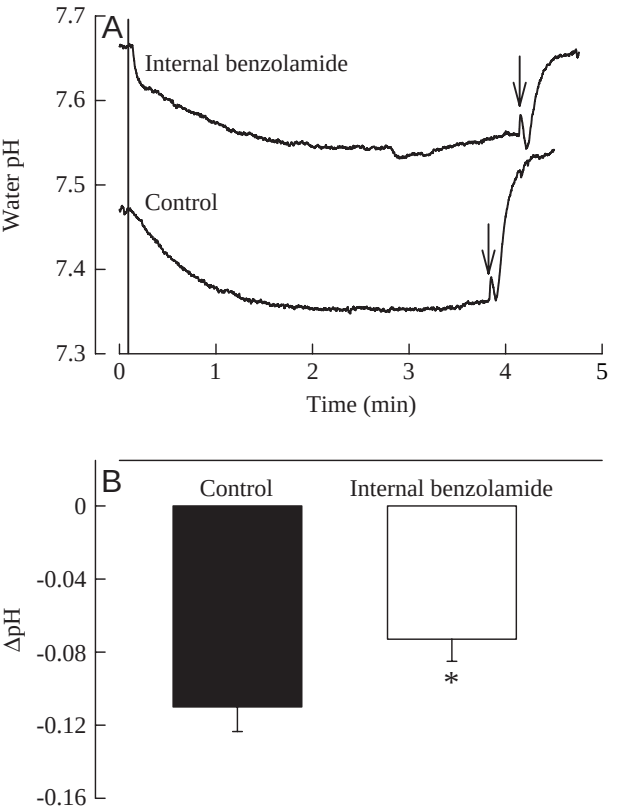

Fig. 5. The effects of internal benzolamide (1.3 mg kg^{-1}) on (A) representative recordings of expired water pH during an approximately 4 min period of stopped flow (bracketed by the two vertical lines and arrows) in dogfish (*Squalus acanthias*) and (B) the mean changes (N=12) in expired water pH (ΔpH) during the stopped-flow period. All other details are as in Fig. 2.

Discussion

Methodology

In the present study, we have used a stopped-flow technique to evaluate the role of external carbonic anhydrase in CO_2 excretion. As discussed in detail by Gilmour (1998), a constant pH during the stoppage of water flow through the measuring chamber indicates that $CO_2/HCO_3^-/H^+$ reactions have reached equilibrium prior to the water arriving at the pH electrode. Such an acid–base equilibrium in expired water, coupled with the establishment of disequilibrium following carbonic anhydrase inhibition, would reveal a role for carbonic anhydrase in catalysing the hydration reaction of excretory CO_2. Conversely, an acidification of expired water during stopped flow would reveal that $CO_2/HCO_3^-/H^+$ reactions had not yet attained equilibrium prior to reaching the pH electrode. Such a disequilibrium pH would suggest that there was inadequate carbonic anhydrase activity available to the expired water to hydrate excretory CO_2 at the fully catalysed rate.

Although simple in theory, there are several practical issues that could potentially confound the interpretation of stopped-flow experiments (see Heming, 1986; Bidani and Heming, 1991; Henry and Heming, 1998; Gilmour, 1998).

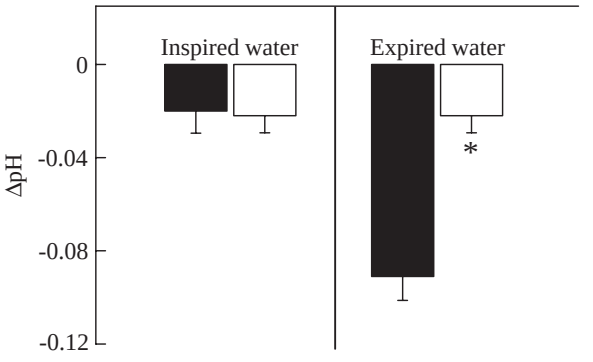

Fig. 6. Changes in pH (ΔpH) in expired and inspired water during 4 min stopped-flow periods in rainbow trout (*Oncorhynchus mykiss*) in the absence (filled columns) or presence (open columns) of external bovine carbonic anhydrase (5 mg l^{-1}). Values are shown as means +1 S.E.M.; N=6. An asterisk indicates a statistically significant difference from the control (no carbonic anhydrase) value (P<0.05).

Importantly, the transit time of the water from the site of CO_2 addition (the gill) to the pH electrode must be sufficiently brief to prevent significant CO_2 hydration from occurring at

the uncatalysed rate. In practice, this is challenging because, unlike the dehydration reaction, the uncatalysed rate of the CO_2 hydration reaction is rapid ($3.5\times10^{-2}\,s^{-1}$ at 25 °C) (Edsall, 1969). Second, the rate of the uncatalysed CO_2 hydration reaction is inversely related to the non-bicarbonate buffering capacity of the expired water. Thus, while the absolute magnitudes of pH disequilibria are increased at low buffering capacities (Bidani and Heming, 1991), their detection is constrained by the rapidity of the uncatalysed CO_2 hydration reaction. In the present study, the buffering capacity of normal sea water was $0.70\,mmol\,l^{-1}\,pH\,unit^{-1}$, and therefore we estimated the half-time of the uncatalysed CO_2 hydration reaction to be approximately 20 s at 11 °C (Henry and Heming, 1998). Therefore, there was probably negligible uncatalysed CO_2 hydration in the expired water prior to stopping the flow given the estimated transit time to the pH electrode (2.4 s). An additional practical consideration is the replacement time for the water flowing through the pH chamber, which must be kept low to ensure that CO_2 hydration is minimised in the water within the pH chamber prior to stopping the flow. In the present study, the inflow:volume ratio of the pH chamber was approximately $200\,min^{-1}$, which would yield a 99 % replacement time of less than 5 s (see Henry and Heming, 1998). Thus, the experimental conditions in the present study were adequate to detect a disequilibrium pH in the expired water. Furthermore, to ensure that pH changes during stopped flow were the result of uncatalysed CO_2 hydration and not an artefact, duplicate experiments were performed on inspired water, which is in acid–base equilibrium and so should not exhibit pH changes during stopped flow.

Dogfish

The expired water of dogfish exhibited a disequilibrium pH, indicating that insufficient carbonic anhydrase activity was accessible to the expired water to bring $CO_2/HCO_3^-/H^+$ reactions to equilibrium. The apparent inadequacy of externally oriented carbonic anhydrase was confirmed by adding exogenous bovine carbonic anhydrase to the water, because this treatment abolished the disequilibrium in the expired water. To determine whether carbonic anhydrase was playing any role in catalysing the hydration of excretory CO_2, the carbonic anhydrase inhibitor acetazolamide was added to the water. The results of this experiment demonstrated that carbonic anhydrase does not contribute to the catalysis of CO_2 hydration in the expired water because the extent of H^+ production during the stoppage of flow was unchanged in the presence of acetazolamide. Thus, while there is morphological evidence that carbonic anhydrase is oriented apically on or within gill epithelial cells (J. Wilson, personal communication), it does not appear to be in contact with the expired water or, if so, is not able to catalyse $CO_2/HCO_3^-/H^+$ reactions. In dogfish, therefore, there is no evidence that external or boundary layer carbonic anhydrase is involved in CO_2 excretion. In the absence of catalysed CO_2 hydration, the blood-to-water P_{CO_2} gradient is presumably being maintained solely by convective removal of excretory CO_2 by gill ventilation.

Although it is widely assumed that the disequilibrium pH in expired water arises from CO_2 that is excreted across the gills, the present study is the first to present direct supporting evidence for this assumption. This was accomplished by using intravascular injection of acetazolamide as a tool to inhibit CO_2 excretion. CO_2 excretion in a variety of fish species is known to be markedly reduced after acetazolamide treatment (e.g. Henry et al., 1988; Dimberg, 1988; Gilmour et al., 1997). Indeed, the results of a parallel study demonstrated an abolition of the arterial–venous difference in blood total CO_2 content of *Squalus acanthias* 30 min after a similar intravascular acetazolamide treatment ($30\,mg\,kg^{-1}$) to that used in the present study. This effect was associated with a reduction of at least 95 % in red blood cell carbonic anhydrase activity (K. M. Gilmour, S. F. Perry, R. Henry, N. Bernier and C. M. Wood, unpublished data). Internal acetazolamide treatment, while significantly reducing the magnitude of the expired water disequilibrium pH, did not eliminate it (Fig. 4). The persistence of an acid–base disequilibrium despite the virtual elimination of red blood cell carbonic anhydrase activity presumably reflects the increasing contribution of transbranchial diffusion of molecular CO_2 as blood P_{CO_2} rises (Gilmour et al., 1997) and the direct addition of CO_2 arising from gill cell metabolism to expired water.

Unlike in teleosts, at least two species of elasmobranch dogfish (*Scyliorhinus canicula* and *Squalus acanthias*) possess carbonic anhydrase activity that is accessible to catalyse plasma $CO_2/HCO_3^-/H^+$ reactions. The catalytic activity originates from carbonic anhydrase circulating within the plasma (Wood et al., 1994; Gilmour et al., 1997; Henry et al., 1997) as well as from gill membrane-bound carbonic anhydrase (Henry et al., 1997), which is believed to be oriented towards the plasma (Gilmour et al., 1997). Although the existence of extracellular carbonic anhydrase in dogfish is not disputed, its potential role in CO_2 excretion has been debated (Wood et al., 1994; Henry et al., 1997; Henry and Heming, 1998; Tufts and Perry, 1998). The results of the present study, however, suggest a significant role for extracellular carbonic anhydrase in CO_2 excretion. Selective inhibition of extracellular carbonic anhydrase activities (plasma and membrane-associated carbonic anhydrase activity) with benzolamide (Swenson and Maren, 1987; Gilmour et al., 1997) caused a significant reduction in the magnitude of the expired water disequilibrium pH (Fig. 5). We believe that this represents a reduced flux of CO_2 across the gill, a consequence of the inhibition of dehydration of HCO_3^- within the plasma. In a parallel study, benzolamide treatment caused a physiologically insignificant inhibition of red blood cell carbonic anhydrase activity (approximately 50 %), yet resulted in a respiratory acidosis and a 30 % reduction in the arterial–venous total CO_2 difference (K. M. Gilmour, S. F. Perry, R. Henry, N. Bernier and C. M. Wood, unpublished data). These data further implicate extracellular carbonic anhydrase activity in CO_2 excretion in dogfish.

Rainbow trout

Immunocytochemical experiments at the electron microscope level (Rahim et al., 1988) have clearly revealed a close association of carbonic anhydrase with the apical membrane of gill pavement and chloride cells. It was suggested that such apical carbonic anhydrase (perhaps trapped within mucus) might be in contact with boundary layer water adjacent to the gill epithelium (Rahim et al., 1988) and could thus play a role in CO_2 excretion. The results of the present study, however, do not support an involvement of external carbonic anhydrase in CO_2 excretion in rainbow trout. As in dogfish, the expired water of trout exhibited a marked disequilibrium pH that was eliminated in the presence of external exogenous bovine carbonic anhydrase. Thus, external carbonic anhydrase, if present, is either inaccessible to expired water or has inadequate activity to catalyse the CO_2 hydration reaction fully.

These results concur with one previous study (Heming, 1986) but conflict with the findings of two others (Wright et al., 1986; Lin and Randall, 1990). As previously discussed (Henry and Heming, 1998; Gilmour, 1998), the contrasting results among the studies probably reflect methodological differences. Wright et al. (1986) performed stopped-flow experiments using poorly buffered ($0.081\,mmol\,l^{-1}\,pH\,unit^{-1}$) Vancouver tap water and a longer replacement time for water in the pH chamber (Henry and Heming, 1998, estimated the replacement time to be greater than 30 s). In such poorly buffered water, the rate of the uncatalysed CO_2 hydration reaction is very rapid (several seconds) and this fact, coupled with a long water replacement time, would make it extremely difficult to measure a disequilibrium pH in expired water. In that same study, the addition of acetazolamide to the water increased the buffering capacity to $0.18\,mmol\,l^{-1}\,pH\,unit^{-1}$ and thus would appreciably slow the rate of the uncatalysed hydration reaction (Bidani and Heming, 1991; Henry and Heming, 1998). This may have been the cause of the disequilibrium pH observed after acetazolamide treatment rather than a specific effect due to inhibition of external carbonic anhydrase activity. Water buffering capacity was not reported in the study of Lin and Randall (1990), but it was presumably also similarly low given that Vancouver tap water (supplemented with NaCl and $CaCl_2$) was used. In the study of Heming (1986) and in the present experiments, the combination of well-buffered water (0.51 and $0.70\,mmol\,l^{-1}\,pH\,unit^{-1}$, respectively) and rapid replacement times for water in the pH chamber (less than 20 s) improved the conditions for the detection of an expired water disequilibrium pH.

The effects of external acetazolamide on the expired water disequilibrium pH of rainbow trout were not assessed in the present study. However, Heming (1986) reported that the addition of acetazolamide to the ambient medium did not affect the disequilibrium pH. Because the addition of acetazolamide presumably increased the buffering capacity of the water in that study, a reduction in the magnitude of the disequilibrium pH would be expected irrespective of whether boundary layer carbonic anhydrase activity is present or not. The fact that the disequilibrium pH was not reduced in the study of Heming (1986) may reflect the opposing effects of increased buffering (reducing the disequilibrium pH) and inhibition of external carbonic anhydrase activity (increasing the disequilibrium pH). An alternative explanation is the potential opposing influences of increased buffering to reduce the disequilibrium pH and a diminished uncatalysed reaction velocity (thus decreasing the extent of pH changes during transit to the pH electrode) to increase the disequilibrium pH. Regardless, unlike in dogfish where a role for external carbonic anhydrase (if present at the gill) in CO_2 excretion has been disproved, further experiments are required before discounting a possible role for externally oriented carbonic anhydrase in rainbow trout.

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