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# CONTRIBUTION OF THE SECONDARY CIRCULATORY SYSTEM TO ACID–BASE REGULATION DURING HYPERCAPNIA IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

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**Summary**

The contribution of the secondary circulatory system to acid–base regulation and epithelial ion transport was evaluated qualitatively in freshwater-acclimated rainbow trout. The dorsal aorta (DA) and the lateral cutaneous vessel (LCV) (which is considered to be the venous drainage of the secondary system) were chronically cannulated and the fish were exposed to environmental hypercapnia (2 % $CO_2$) after establishment of normocapnic control values. Fluid sampled from the LCV contained much less haemoglobin (0.14 g 100 $ml^{-1}$) and fewer blood cells (packed cell volume, PCV, 1.2–1.7 %) than DA blood ([Hb] 8.2–8.9 g 100 $ml^{-1}$, PCV 27.2–32.5 %) regardless of ambient $CO_2$ levels, indicating highly limited access of red blood cells to the secondary circulatory system through anastomoses connecting it to the primary system. There was no significant difference between the two sampling sites for any of the acid–base variables (pH, $P_{CO_2}$, [$HCO_3^-$]) and most plasma ion concentrations ([$Na^+$], [$Ca^{2+}$], [$Mg^{2+}$] and [$Cl^-$]) during normocapnia, although plasma [$K^+$] and osmolarity were significantly lower in LCV fluid. Upon exposure to hypercapnia, the pH of LCV fluid became significantly higher than that of DA plasma because of the higher [$HCO_3^-$] in the LCV plasma than in the DA plasma. The LCV plasma concentration of $Cl^-$ was significantly lower than that of DA plasma during hypercapnia. These data suggest that the secondary circulatory system draining the body surface is involved in ionic acid–base regulation in fish, probably *via* $Cl^-/HCO_3^-$ exchange processes across the extrabranchial epithelium of the body surface.

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Key words: secondary circulatory system, acid–base regulation, ionic transfer, body surface epithelium, rainbow trout, *Oncorhynchus mykiss*.

## Introduction

Recent anatomical studies have cast doubts on the nature of the so-called lymphatic system in fish. Close inspection of vascular casts of five species of fish (Vogel, 1981; Vogel and Claviez, 1981) as well as *in vivo* observation of the microcirculatory system in the transparent glass catfish (Steffensen *et al.* 1986) revealed the so-called lymphatic vessels to be the drainage conduits of a unique vascular system, designated by Vogel (1981) as the 'secondary circulatory system'. Flow into the secondary system originates from arteries of the primary circulatory system *via* numerous capillary-sized (inner diameter 10–15 $\mu$m) anastomoses (Steffensen *et al.* 1986). The secondary system itself consists of distributing, exchange and collecting vessels analogous to the arteries, capillaries and veins of the primary system, respectively. Capillaries of the secondary system appear to be distributed mainly in the internal and external body surfaces, such as the mucosa of the skin and pharynx, as well as the peritoneum. Vogel (1985) demonstrated that dense networks of secondary capillaries exist above the scales, directly underlying the skin epithelium of trout and tilapia. Sixteen years earlier, an intricate capillary network beneath the epidermis of the exposed part of each scale was described for pike by Tysekiewicz (1969); a system probably analogous to the superficial secondary capillaries described by Vogel (cf. Satchell, 1991).

Based on the distribution of secondary capillaries, Vogel (1985) and Satchell (1991) have suggested that the secondary system may play a role in transepithelial ion transport. The strikingly close juxtaposition between the large surface area of skin secondary capillaries with the epithelium and the external environment implies strongly that the body surface area is a potentially important site for ion and acid–base regulation. The lack of any relevant physiological studies on this subject may be related to the methodological difficulties in isolating the fluid from the secondary system. The aim of the present study was to compare acid–base status and ion concentrations of the secondary vascular system fluid with those of blood plasma from the primary circulatory system at rest and during acid–base disturbance. For this purpose, the dorsal aorta (DA) and the lateral cutaneous vessel of the secondary vascular system (LCV, 'lateral lymphatic' according to Kampmeier, 1969) were chronically cannulated in rainbow trout (*Oncorhynchus mykiss*). Animals were, after a normocapnic control period, subjected to environmental hypercapnia in order to elucidate the contribution of the secondary vascular system to the regulation of acid–base homeostasis during this state of stimulated transepithelial ion transport.

## Materials and methods

### *Experimental animals*

Rainbow trout [*Oncorhynchus mykiss* (Walbaum)] were obtained from a hatchery near Köln (Germany), and kept for several weeks to several months in large, well-aerated glass aquaria (2–3 $m^3$) at 15 °C with a continuous supply of dechlorinated and aerated Göttingen tap water (100–200 ml $fish^{-1}$ $min^{-1}$; ionic

composition: $[Na^+]$ 0.35–0.4 mmol $l^{-1}$, $[Cl^-]$ 0.3–0.35 mmol $l^{-1}$, $[HCO_3^-]$ 0.8–1.2 mmol $l^{-1}$, $[Ca^{2+}]$ 0.7–1 mmol $l^{-1}$, pH 7.7–8.1). Body mass ranged from 675 to 1150 g. The fish were maintained on a commercial trout diet until 2 days prior to surgery.

### *Surgery*

The fish were anaesthetized by immersion in a solution of 0.01 % MS-222 in Göttingen tap water, buffered by addition of $NaHCO_3$. During surgery, the gills were irrigated to maintain anaesthesia with a solution of 0.005 % MS-222 solution, bubbled with pure $O_2$ and thermostatted to 15 °C.

The dorsal aorta (DA) was cannulated with PE50 catheters by application of a modified Seldinger technique, similar to the procedure of Soivio *et al.* (1975). Following DA cannulation, the LCV was non-occlusively cannulated. The vessel was exposed by a short (7–10 mm) dorso-ventral incision in the skin, slightly dorsal to the lateral line at the level of the rostral margin of the dorsal fin. By careful dissection and retraction, the vessel was exposed with very little damage to the subcutaneous tissue. T-pieces, constructed from PE50, were inserted into the LCV. Both tips of the horizontal bar of the T-piece were covered with silastic tubing of the same diameter to facilitate insertion. The wound was closed with fine atraumatic silk sutures (6/0). The cannulae were filled with heparinized (50 i.u. $ml^{-1}$) Cortland saline (Wolf, 1963) without glucose or with similarly heparinized 0.9 % NaCl solution. Surgery took about 20 min.

After surgery, the fish were allowed to recover for at least 20 h in a recirculating water system consisting of a fish chamber and a 200 l reservoir. The water in the reservoir was vigorously aerated, thermostatted to 15 °C, and circulated through the chamber at a flow rate of 5 l $min^{-1}$. The fish chamber was darkened to prevent visual disturbance to the fish.

### *Protocol*

After two control samples had been taken, 3 h apart, the system was equilibrated with 2 % $CO_2$ (water $P_{CO_2}$ stabilized at the new level within 5–7 min due to counter-current water/gas flow in our system, vigorous aeration of 7–12 l $min^{-1}$ and the high recirculation rate), and subsequent samples were taken 1 and 4 h after the onset of hypercapnia. For DA sampling, the first 1 ml was withdrawn with a syringe to clear the cannula of saline and the following 800 $\mu$l was collected in an Eppendorf vial by siphoning. The vial was centrifuged at 16 000 *g* to obtain plasma for ion analyses. LCV fluid was collected by siphoning, discarding the first 1 ml to clear the cannula and collecting the next 800 $\mu$l, which was treated in the same way as the DA samples. Two microhaematocrit tubes were filled from each cannula for acid–base measurements.

### *Analytical methods*

pH was determined using a glass microcapillary electrode thermostatted to the animal's body temperature and standardized with precision phosphate buffers

(Radiometer BMS3 Mk2). Samples of blood and LCV fluid were centrifuged anaerobically at 16000 *g* and total $CO_2$ ($T_{CO_2,pl}$) of the supernatant was determined by measurement of differential conductivity, utilizing a Capnicon III instrument (Cameron Instruments, Port Aransas, TX, USA). The partial pressure of $CO_2$ ($P_{CO_2}$) in the plasma was calculated from measured pH and $T_{CO_2}$ values applying the Henderson–Hasselbalch equation with appropriate constants (Heisler, 1984, 1990) (note: the last line term of the formula for $CO_2$ solubility in Heisler, 1984, is misprinted and should read '+'). Plasma $[HCO_3^-]$ was calculated by subtracting the fraction of molecular $CO_2$ from $T_{CO_2}$. Haemoglobin concentration ([Hb]) was determined by the cyanmethaemoglobin method (Sigma Diagnostics kit no. 525). Packed cell volume (PCV: haematocrit plus white cells) was measured after centrifugation at 16000 *g* for 5 min. Osmolarity was determined with a Wescor 5100C vapour pressure osmometer. Plasma concentrations of sodium ($[Na^+]_{pl}$), potassium ($[K^+]_{pl}$), magnesium ($[Mg^{2+}]_{pl}$) and calcium ($[Ca^{2+}]_{pl}$) were measured by atomic absorption spectrophotometry (Perkin-Elmer model 2380), and plasma chloride concentration ($[Cl^-]_{pl}$) was determined by coulometric titration (digital chloride titrator; Haake Buchler Instruments, New Jersey, USA). Plasma protein concentration ($[protein]_{pl}$) was analyzed colorimetrically (Sigma Diagnostics kit no. 540). DA and LCV pressures were continuously recorded between samples using blood pressure transducers (Statham P23PB) and a chart recorder (Gould 2600S).

### *Statistical analysis*

In order to circumvent the large interindividual variability a paired *t*-test was used for comparison between data taken simultaneously from the two sampling sites (DA and LCV) (cf. Zar, 1984). Statistical differences between values obtained during control and hypercapnic periods were assessed for each site by the method of Dunnett (1964), the method of choice suggested by Wallenstein *et al.* (1980). Data are generally expressed as mean±S.E.

## Results

### *Control condition*

Control values of 12 measured variables in DA blood and LCV fluid are summarized in Table 1. Although all of the measured variables tended to differ between the two sites, only PCV, [Hb] (Fig. 1), $[K^+]_{pl}$ (Fig. 3), and osmolarity were significantly different. Since LCV fluid contained very few red blood cells, a large fraction of the packed cell volume presented in Table 1 is attributable to white blood cells. $P_{CO_2}$ and $[HCO_3^-]_{pl}$ of the fish used in the present study were high for rainbow trout. Data were confirmed repeatedly by different operators using a number of instruments. Since similar data were also obtained from some fish with only DA cannulae, the LCV cannulation apparently did not affect the acid–base and ionic status of the animal to any significant extent. Thus, the

Table 1. *Comparison of measured variables between the dorsal aortic blood plasma (DA) and lateral vessel fluid (LCV) of freshwater-acclimated rainbow trout under control conditions*

| Variable | Units | DA | LCV | Significance |
|---|---|---|---|---|
| Acid–base status | | | | |
| pH | | 8.080±0.064 | 7.942±0.089 | |
| $P_{CO_2}$ | (kPa) | 0.55±0.03 | 0.87±0.11 | |
| | (mmHg) | 4.1±0.22 | 6.5±0.86 | |
| $[HCO_3^-]_{pl}$ | (mmol $l^{-1}$) | 20.6±3.19 | 21.2±3.8 | |
| Haematology | | | | |
| Packed cell volume | (%) | 27.2±1.92 | 1.7±0.33 | $P<0.01$ |
| [Hb] | (g 100 $ml^{-1}$) | 8.2±0.58 | 0.14±0.02 | $P<0.01$ |
| $[Protein]_{pl}$ | (g 100 $ml^{-1}$) | 5.9±0.87 | 3.7±0.39 (5) | |
| Ionic status | | | | |
| $[Na^+]_{pl}$ | (mmol $l^{-1}$) | 144.8±3.43 | 143.9±4.0 | |
| $[K^+]_{pl}$ | (mmol $l^{-1}$) | 3.5±0.16 | 2.4±0.18 | $P<0.01$ |
| $[Ca^{2+}]_{pl}$ | (mmol $l^{-1}$) | 2.3±0.17 | 2.2±0.12 | |
| $[Mg^{2+}]_{pl}$ | (mmol $l^{-1}$) | 0.81±0.03 | 0.80±0.02 (5) | |
| $[Cl^-]_{pl}$ | (mmol $l^{-1}$) | 117.1±4.86 | 115.3±4.5 | |
| Plasma osmolarity | (mosmol $l^{-1}$) | 284.2±2.73 | 277.9±0.6 | $P<0.05$ |

Values are mean±S.E., $N=6$ unless given in parentheses.
Significant difference between the LCV value and the corresponding DA value are calculated by paired *t*-test.

somewhat unusually high $[HCO_3^-]$ and $P_{CO_2}$ values have to be attributed to strain variability or to a hypercapnic state of unknown cause.

### *Response to hypercapnia*

As a characteristic response, the PCV of DA blood increased significantly after exposure to hypercapnia, whereas the simultaneous rise in [Hb] and the fall in [protein] were statistically insignificant (Fig. 1). Hypercapnia did not affect the significance of differences between DA and LCV values for PCV and [Hb]. During hypercapnia, pH and $[HCO_3^-]_{pl}$ of LCV fluid became significantly higher than the corresponding values for DA blood, although the $[HCO_3^-]_{pl}$ difference was statistically significant only after 4 h of hypercapnia (Fig. 2). There were no differences in $P_{CO_2}$ between sample sites. After the onset of hypercapnia, the LCV $[Cl^-]_{pl}$ became significantly lower than the DA plasma concentration, whereas $[Na^+]_{pl}$ and $[K^+]_{pl}$ remained essentially unaffected (Fig. 3). $[Mg^{2+}]_{pl}$ was slightly, but significantly, increased at both sites (approximately 0.1 mmol $l^{-1}$) after 4 h, but there was no significant difference between sites. A significant difference in $[K^+]_{pl}$ between sites under control conditions vanished upon exposure to hypercapnia, and the DA concentration was significantly decreased at 1 h of hypercapnia. $[Ca^{2+}]_{pl}$ was unaffected by hypercapnia, indicating, together with the data on $[Mg^{2+}]$, that osteal structures were not involved in the accumulation of $HCO_3^-$

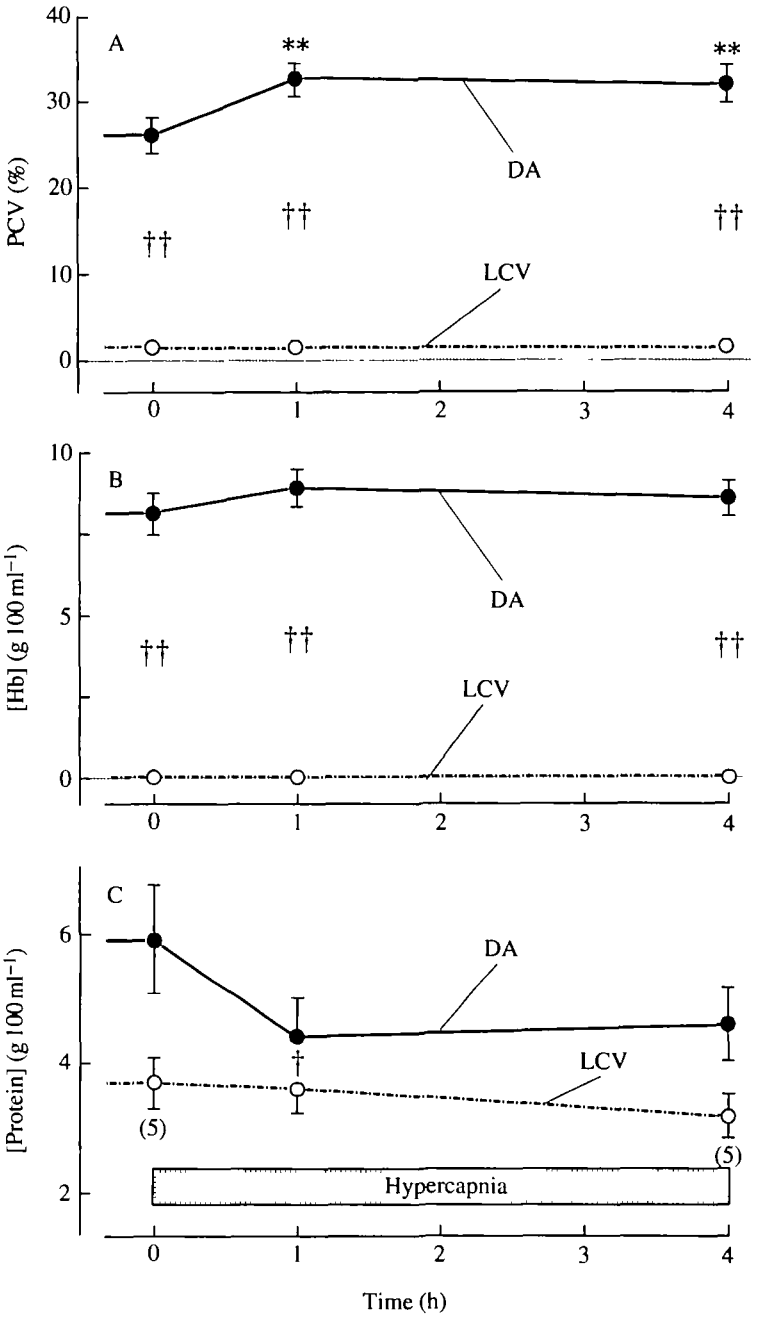


Fig. 1

during hypercapnia. Osmolarity in the DA fell slightly during hypercapnia, eliminating a small but significant difference from that of the LCV found under control conditions.

Fig. 1. Packed cell volume (PCV), haemoglobin ([Hb]) and plasma protein concentration in dorsal aortic blood (DA) and lateral cutaneous vessel fluid (LCV) of freshwater rainbow trout during environmental hypercapnia (2 % $CO_2$). Mean±S.E., $N$=6 (unless otherwise indicated). Asterisks indicate significantly different values from the corresponding controls at the same site (** $P<0.01$, Dunnett's test). Daggers indicate a significant difference between the two sites at corresponding times († $P<0.05$, †† $P<0.01$, paired $t$-test).

Hypercapnia did not significantly affect cardiovascular variables. Heart rate ($f_H$) and average DA blood pressure ($P_{DA}$) were the same under control and hypercapnic periods [$f_H$ 64.7±6.5 and 61.6±6.5 $min^{-1}$; $P_{DA}$ 41.2±1.7 and 41.4±2.0 $cmH_2O$ (0.404±0.017 and 0.406±0.20 kPa), $N$=5, respectively]. LCV pressure was very low (−1.3 to 3.8 $cmH_2O$; −0.2 to 0.5 kPa) and somewhat variable irrespective of $CO_2$ levels.

## Discussion

The important role of transepithelial ion transfer for acid–base regulation in fish is well established. Electroneutral $Cl^-/HCO_3^-$ and $Na^+/NH_4^+(H^+)$ exchange mechanisms are generally accepted as the mechanisms predominantly responsible for compensation of exogenous and endogenous acid–base perturbations (Evans, 1986; Heisler, 1986*a*), but an electrogenic transepithelial $H^+$ pump has recently been postulated to be involved as well (Avella and Bornancin, 1989; Lin and Randall, 1991). Although a general understanding of the ion movements during acid–base regulation in fish has been established during the past ten years, our knowledge of the underlying physiological mechanisms is incomplete, and the amount of information concerning sites of transepithelial transfers remains scant. A number of studies have been conducted to evaluate the role of the kidneys in fish acid–base regulation and these have generally indicated a minor contribution (for a review, see Heisler, 1986*a*). The gills, with their large epithelial surface area, short blood–water distances and high rates of perfusion and ventilation, are accordingly thought to be primarily responsible for the observed ion transfers. A possible contribution of cutaneous exchange sites has been assessed to be minor (<1 %) in at least one species of elasmobranch fish (Heisler *et al.* 1976), but analogous experiments have never been performed in teleosts.

Fish skin is generally considered to be poorly permeable to ions and water (Fromm, 1968), but a few studies have indicated a significant role of the skin for transfer of ions not related to acid–base regulation. In resting freshwater rainbow trout about 50 % of the $Ca^{2+}$ uptake takes place over the general body surface (Perry and Wood, 1985), and the $Cl^-$ efflux through the skin of the seawater shanny (*Blennius pholis*) accounted for 65 % of the total efflux (Nonnotte *et al.* 1979). An earlier study by Mashiko and Jozuka (1964) demonstrated that the fins were involved in $Ca^{2+}$ uptake and release, but more so in a seawater species (*Duymaeria flagellifera*) than in a freshwater species (*Carassius carassius*).

Chloride cells are considered to be responsible for most of the transepithelial

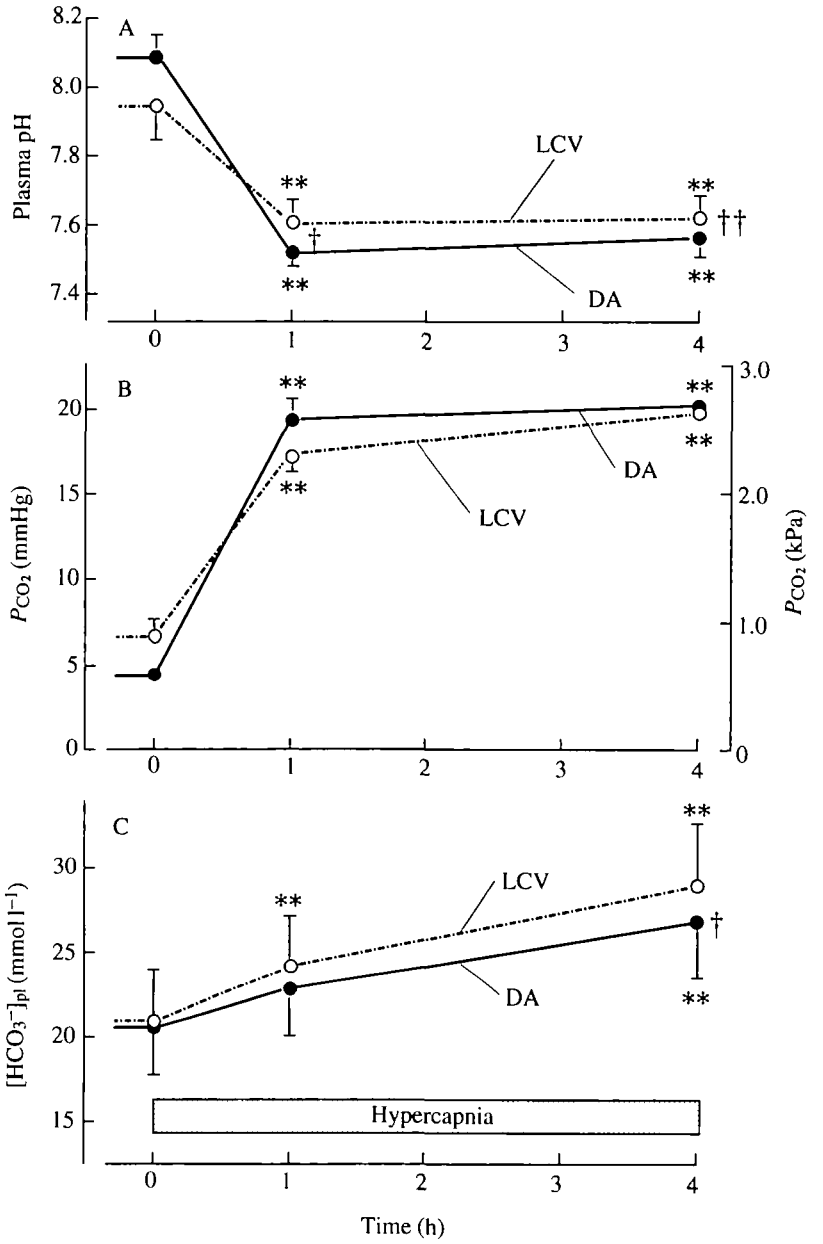


Fig. 2. Plasma pH, $P_{CO_2}$ and $[HCO_3^-]$ in dorsal aortic blood (DA) and lateral cutaneous vessel fluid (LCV) of freshwater rainbow trout during environmental hypercapnia (2% $CO_2$). Mean±S.E., $N$=6. Asterisks denote significantly different values from the corresponding controls at the same site (** $P<0.01$, Dunnett's test). Daggers denote a significant difference between the two sites at corresponding times († $P<0.05$, †† $P<0.01$, paired $t$-test).

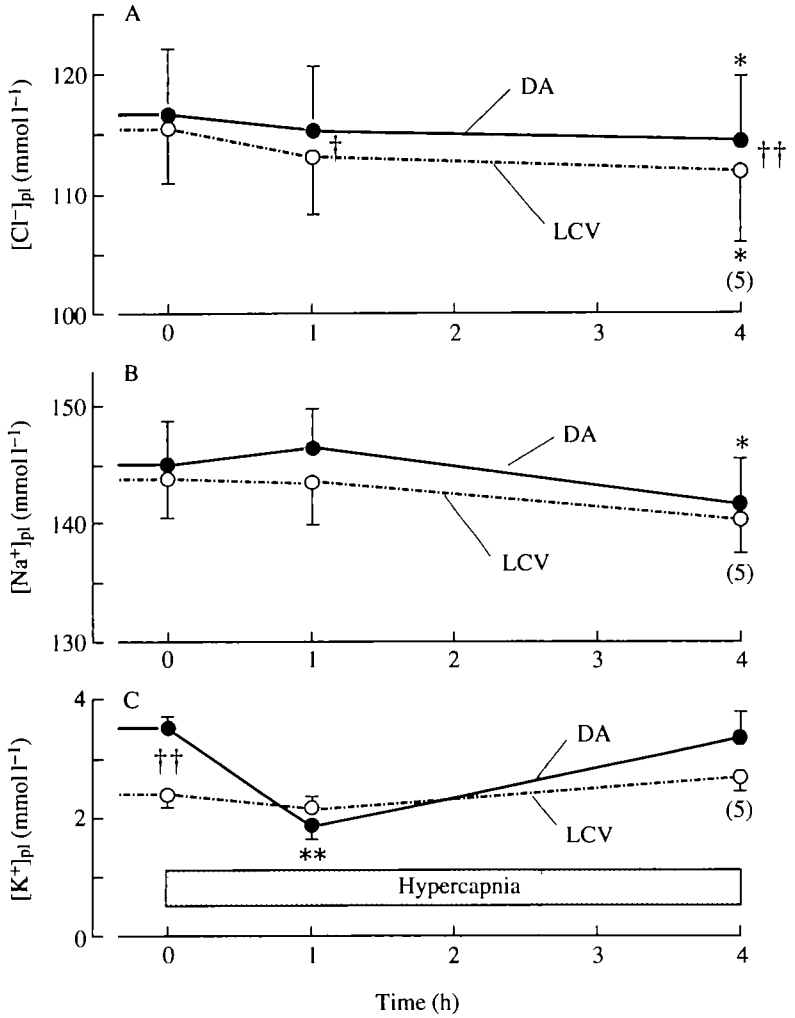


Fig. 3. Plasma $[Cl^-]$, $[Na^+]$ and $[K^+]$ in dorsal aortic blood (DA) and lateral cutaneous vessel fluid (LCV) of freshwater rainbow trout during environmental hypercapnia (2% $CO_2$). Mean±S.E., $N=6$ (unless otherwise indicated). Asterisks denote significantly different values from the corresponding controls at the same site (* $P<0.05$, ** $P<0.01$, Dunnett's test). Daggers denote significant differences between the two sites at corresponding times († $P<0.05$, †† $P<0.01$, paired $t$-test).

ion transfer and they occur in the skin of several fish species (Hwang, 1989; Henrikson and Matoltsy, 1968; Stiffler *et al.* 1986; Nonnotte *et al.* 1979), suggesting that fish skin may be involved in the ion exchange processes required for acid–base homeostasis. In special cases, chloride cells occur in large numbers in specialized body surface areas. The opercular epithelium of *Fundulus heteroclitus* (Karnaky and Kinter, 1977) and *Sarotherodon mossambicus* (Foskett *et al.* 1981) and the skin of *Gillichthys mirabilis* (Marshall, 1977) are such sites, which have been shown by

*in vitro* studies to be the location of high ion transfer activity. However, the serosal environment of those rich chloride cell populations must be in very close proximity to the vasculature of the secondary circulatory system to allow for effective exchange between the interstitial fluid and the bulk extracellular fluid. Such an arrangement is seen in the gills, where the central venous sinus directly underlies the filamental epithelium containing the chloride cells.

Although the juxtaposition of chloride cells and secondary circulation has not yet been demonstrated, the abundance of ion-transporting epithelial cells on the body surface would be a rather ineffective system of ion transfer without the support of an underlying functional convective system. A relatively high rate of convective mixing will be a critical factor for an effective link between ion-transferring epithelia and the bulk extracellular fluid. This is particularly important with respect to the establishment of ionic gradients. A low rate of convective mixing will result in a build-up of ions in the interstitial fluid, reducing the ion transfer velocity by increased electrochemical gradients, or it may even effect complete shut-down of any transfer until the accumulated ions are removed by convective mechanisms, similar to those observed during lactacidosis in skeletal muscle tissue (perfusion/equilibrium limitation; Heisler, 1986*b*). Perfusion of the secondary vascular system with 'skimmed' blood containing very few red cells may provide a much more effective transport vehicle, because of the lower viscosity of LCV fluid. This will result in higher flow rates at lower perfusion pressures, providing a considerable saving of cardiac energy (Satchell, 1991). As observed by Vogel (1985), and shown in this study, the fluid from the secondary system has a low haematocrit, supporting this view of perfusion energetics. Similar differences, although not as pronounced, have been reported between the ventral aorta and the subcutaneous sinus of hagfish, and this has been postulated to be the evolutionary forerunner of the cutaneous vessels in teleosts (Forster *et al.* 1989). However, transport of ions is energetically expensive, and tissues involved in ionic regulation are generally characterized by a substantial metabolic rate. Since fluid pumped through the secondary system will, owing to the lack of red blood cells, not provide any reasonable transport medium for oxygen, oxygen has to be taken up directly from the ambient water. Fish skin consumes a considerable amount of oxygen directly from the environment (Kirsch and Nonotte, 1977; Nonotte and Kirsch, 1978; for a review, see Feder and Burggren, 1985), indicating a high metabolic activity probably related to ion transfer processes.

The secondary circulatory system originates from the arterial side of the primary system and, therefore, the ionic composition of the secondary arterial plasma should be identical to that of the arterial plasma in the primary circulation, as long as ionic exchange with the environment does not take place. In this study, we have collected samples from the lateral cutaneous vessel, which is regarded as the venous drainage of the secondary system. Although we have not examined the direction of fluid flow in the LCV, its open connection with the common cardinal vein at the rostral end of the LCV (Kampmeier, 1969; Wardle, 1971) and the near absence of red blood cells in LCV fluid (Table 1, Fig. 1) are compatible only with

forward flow in the vessle, at least in its anterior portion. Accordingly, any difference in acid–base and ionic status from the fluid of the primary system (DA plasma) should reflect ion exchange processes through the capillary walls of the secondary circulatory system. We postulate that the LCV would receive fluid collected exclusively from the segmental veins of the secondary system after it has traversed the capillary networks in the skin. Under these conditions, differences in ionic and acid–base composition have to be ascribed to transcutaneous transport. A possible small admixture of fluid from deep secondary vessels, however, cannot be ruled out.

During normocapnia, acid–base status and electrolyte concentrations were almost identical at the two sites, except for $[K^+]_{pl}$ and osmolarity (Table 1). The basis for these differences is unclear and the mean difference in osmolarity ($6\,mosmol\,l^{-1}$) cannot be explained by the difference in $[K^+]_{pl}$ alone. Thus, under control conditions the skin of trout does not appear to be significantly involved in ion transfer as far as net ion fluxes are concerned. In contrast, hypercapnia induced a significant difference in pH between the two sites (LCV pH > DA pH) in spite of identical $P_{CO_2}$ values (Fig. 2). This was brought about by a higher LCV $[HCO_3^-]_{pl}$. Based on the average difference of $2.1\,mmol\,l^{-1}$ between the DA and LCV, and the amount of $HCO_3^-$ produced by non-bicarbonate buffering due to the shift in pH ($0.1\,mmol\,l^{-1}$, buffer line according to Wood *et al.* 1982), about $2.2\,mmol\,l^{-1}$ of bicarbonate has been gained after 4 h of hypercapnia. This concentration difference corresponds very well with the reduction in $[Cl^-]_{pl}$ ($2.4\,mmol\,l^{-1}$) (Fig. 3). This suggests a net $Cl^-/HCO_3^-$ exchange in the secondary circulatory system of the body surface. A similar relationship between positive $[HCO_3^-]$ differences and negative $[Cl^-]$ differences has repeatedly been observed in plasma of fish during exposure to hypercapnia (Toews *et al.* 1983; Perry *et al.* 1987; Cameron and Iwama, 1987; Iwama and Heisler, 1991; G. K. Iwama, A. Ishimatsu and N. Heisler, in preparation), whereas the effect of hypercapnia on other plasma ion concentrations is less consistent.

Although the above data strongly suggest the involvement of the secondary circulatory system in the acid–base regulation during hypercapnia, its quantitative role in the overall process may not be large. Recently, Steffensen and Lomholt (1992) estimated the perfusion rate in the secondary system based on data from the distribution of labelled plasma proteins between the primary and secondary systems to be $6.6\,ml\,kg^{-1}\,h^{-1}$. This is equivalent to 0.3 % of cardiac output. This may indicate a rather limited physiological significance of the epithelial ionic transport mediated by the secondary circulatory system. On the basis of this estimate of perfusion rate and the bicarbonate concentration difference reported in this study, the $HCO_3^-$ gain through the secondary system would amount to only $0.05\,mmol\,kg^{-1}$ for 4 h of hypercapnia, which represents, in relation to the total extracellular gain during 4 h of $1.26\,mmol\,kg^{-1}$ ($\Delta[HCO_3^-]_{DA}$ × relative extracellular volume = $6.3\times0.2\,mmol\,kg^{-1}$), a contribution of only 4 %. However, it must be kept in mind that the above perfusion rate has been obtained from resting rather than hypercapnia-stressed fish. Recent data from our experiments indicate

that the gill filamental epithelium overlying the central venous sinus accounts for a major fraction of the bicarbonate gained from the environment during hypercapnia in rainbow trout (G. K. Iwama, A. Ishimatsu and N. Heisler, in preparation); such data certainly diminish the possibility of a large contribution from the secondary circulation system at the body surface. However, the estimated relative contribution is not in accordance with the high rate of skin oxygen consumption, which can only be attributed to ion transport. Also, the magnitude of diffusional ion exchange between the secondary and primary (venous) systems is still unknown. Further studies are required for a quantitative evaluation of the role of this subsystem in ionic and acid–base regulation.

Determination of LCV flow using labelled proteins may not be an appropriate method for measuring 'effective LCV convection'. We injected Methylene Blue into the secondary system of one anaesthetized trout *via* a cannula occlusively inserted into the caudal part of the lateral cutaneous vessel at the level of the pectoral fin. On dissection, it became evident that the dye was almost exclusively located in the kidneys. This is in accordance with the report of Kampmeier (1969) stating that the LCV of teleosts is connected to the deep 'lymphatics' running alongside the kidneys at the level of the caudal extremity of the abdominal cavity (called the 'circumanal lymph plexus'). Flow of the dye from the lateral cutaneous vessel through the circumanal plexus to the kidneys indicates the path of least resistance. Unfortunately, the natural direction of flow through the plexus, whether from the kidneys to the LCV or in the opposite direction, is unknown, as is the connection or distribution of the secondary system within the capillary networks of the kidneys. The vascular connection through the circumanal plexus may represent a functional linkage between the kidneys and the secondary surface capillary bed. The role of the secondary system may be that of a more or less closed secondary convective pool, facilitating transfer or ions from the fluid bathing the ion-transporting cells of the body surface to the primary system. Flow in this system may be induced by pulsations of the primary vasculature, by local pressure changes induced by muscular activity, and/or by the activity of lymph hearts. Convective mixing of secondary vascular fluid between the body surface and the kidneys could affect the composition of the extratubular interstitial fluid, thus providing a special extracellular environment for ion-transporting kidney cells. The close relationship between the secondary circulation and the kidney tissues may accordingly allow for elimination of protons to (or gain of bicarbonate from) the secondary system with modifications of renal effluent plasma that are not reflected by changes in urine composition. With such an interrelationship between the primary and secondary systems, the kidneys may have a much larger role in acid–base regulation than thought previously. An evaluation of their physiological role by urinary catheter alone may prove to be inadequate.

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## References

AVELLA, M. AND BORNANCIN, M. (1989). A new analysis of ammonia and sodium transport through the gills of the freshwater rainbow trout (*Salmo gairdneri*). *J. exp. Biol.* **142**, 155–175.

CAMERON, J. N. AND IWAMA, G. K. (1987). Compensation of progressive hypercapnia in channel catfish and blue crabs. *J. exp. Biol.* **133**, 183–197.

DUNNETT, C. W. (1964). New tables for multiple comparisons with a control. *Biometrics* **20**, 482–491.

EVANS, D. H. (1986). The role of branchial and dermal epithelia in acid–base regulation in aquatic vertebrates. In *Acid–Base Regulation in Animals* (ed. N. Heisler), pp. 139–172. Amsterdam, Oxford: Elsevier.

FEDER, M. E. AND BURGGREN, W. W. (1985). Cutaneous gas exchange in vertebrates: Design, patterns, control and implications. *Biol. Rev.* **60**, 1–45.

FORSTER, M. E., DAVISON, W., SATCHELL, G. H. AND TAYLOR, H. H. (1989). The subcutaneous sinus of the hagfish, *Eptatretus cirrhatus* and its relation to the central circulating blood volume. *Comp. Biochem. Physiol.* **93**A, 607–612.

FOSKETT, J. K., LOGSDON, C. D., TURNER, T., MACHEN, T. E. AND BERN, H. A. (1981). Differentiation of the chloride extrusion mechanism during seawater adaptation of a teleost fish, the cichlid *Sarotherodon mossambicus*. *J. exp. Biol.* **93**, 209–224.

FROMM, P. O. (1968). Some quantitative aspects of ion regulation in teleosts. *Comp. Biochem. Physiol.* **27**, 865–869.

HEISLER, N. (1984). Acid–base regulation in fishes. In *Fish Physiology*, vol. X A (ed. W. S. Hoar and D. J. Randall), pp. 315–401. New York, London: Academic Press.

HEISLER, N. (1986*a*). Acid–base regulation in fishes. In *Acid–Base Regulation in Animals* (ed. N. Heisler), pp. 309–356. Amsterdam, Oxford: Elsevier.

HEISLER, N. (1986*b*). Comparative aspects of acid–base regulation. In *Acid–Base Regulation in Animals* (ed. N. Heisler), pp. 397–450. Amsterdam, Oxford: Elsevier.

HEISLER, N. (1990). Acid–base regulation: Interrelationships between gaseous and ionic exchange. In *Vertebrate Gas Exchange from Environment to Cell* (ed. R. G. Boutilier), pp. 211–251. In *Advances in Environmental and Comparative Physiology*, vol. 6 (managing editor, R. Gilles). Heidelberg, Berlin, New York: Springer.

HEISLER, N., WEITZ, H. AND WEITZ, A. M. (1976). Hypercapnia and resultant bicarbonate transfer processes in an elasmobranch fish. *Bull. Eur. Physiopathol. Respir.* **12**, 77–85.

HENRIKSON, R. C. AND MATOLTSY, A. G. (1968). The fine structure of teleost epidermis. III. Club cells and other cell types. *J. Ultrastruct. Res.* **21**, 222–232.

HWANG, P. P. (1989). Distribution of chloride cells in teleost larvae. *J. Morph.* **200**, 1–8.

IWAMA, G. K. AND HEISLER, N. (1991). Effect of environmental water salinity on acid–base regulation during environmental hypercapnia in the rainbow trout (*Oncorhynchus mykiss*). *J. exp. Biol.* **158**, 1–18.

KAMPMEIER, O. F. (1969). *Evolution and Comparative Morphology of the Lymphatic System.* Illinois: Charles C. Thomas.

KARNAKY, K. J., JR AND KINTER, W. B. (1977). Killifish opercular skin: a flat epithelium with a high density of chloride cells. *J. exp. Zool.* **199**, 355–364.

KIRSCH, R. AND NONOTTE, G. (1977). Cutaneous respiration in three freshwater teleosts. *Respir. Physiol.* **29**, 339–354.

LIN, H. AND RANDALL, D. J. (1991). Evidence for the presence of an electrogenic proton pump on the trout gill epithelium. *J. exp. Biol.* **161**, 119–134.

MARSHALL, W. S. (1977). Transepithelial potential and short-circuit current across the isolated skin of *Gillichthys mirabilis* (Teleostei: Gobiidae), acclimated to 5% and 100% seawater. *J. comp. Physiol.* **114**B, 157–165.

MASHIKO, K. AND JOZUKA, K. (1964). Absorption and excretion of calcium by teleost fishes with special reference to routes followed. *Ann. Zool. Japon.* **37**, 41–50.

NONOTTE, G. AND KIRSCH, R. (1978). Cutaneous respiration in seven sea-water teleosts. *Respir. Physiol.* **35**, 111–118.

NONNOTTE, G., NONNOTTE, L. AND KIRSCH, R. (1979). Chloride cells and chloride exchange in the skin of a sea-water teleost, the shanny (*Blennius pholis* L.). *Cell Tissue Res.* **199**, 387–396.

PERRY, S. F., MALONE, S. AND EWING, D. (1987). Hypercapnic acidosis in the rainbow trout (*Salmo gairdneri*). I. Branchial ionic fluxes and blood acid–base status. *Can. J. Zool.* **65**, 888–895.

PERRY, S. F. AND WOOD, C. M. (1985). Kinetics of branchial calcium uptake in the rainbow trout: effects of acclimation to various external calcium levels. *J. exp. Biol.* **116**, 411–433.

SATCHELL, G. H. (1991). *Physiology and Form of Fish Circulation.* Cambridge: Cambridge University Press.

SOIVIO, A., NYHOLM, K. AND WESTMAN, K. (1975). A technique for repeated sampling of the blood of individual resting fish. *J. exp. Biol.* **62**, 207–217.

STEFFENSEN, J. F. AND LOMHOLT, J. P. (1992). The secondary vascular system. In *Fish Physiology*, vol. 12, *Cardiovascular System* (ed. W. S. Hoar, D. J. Randall and A. P. Farrell). Orlando: Academic Press (in press).

STEFFENSEN, J. F., LOMHOLT, J. P. AND VOGEL, W. O. P. (1986). *In vivo* observations on a specialized microvasculature, the primary and secondary vessels in fishes. *Acta zool. Stockh.* **67**, 193–200.

STIFFLER, D. F., GRAHAM, J. B., DICKSON, K. A. AND STOCKMANN, W. (1986). Cutaneous ion transport in the freshwater teleost *Synbranchus marmoratus*. *Physiol. Zool.* **59**, 406–418.

TOEWS, D. P., HOLETON, G. F. AND HEISLER, N. (1983). Regulation of the acid–base status during environmental hypercapnia in the marine teleost fish *Conger conger*. *J. exp. Biol.* **107**, 9–20.

TYSEKIEWICZ, K. (1969). Structure and vascularization of the skin of the pike (*Esox lucius* L.). *Acta biol. cracoviensia., Zoologia* **12**, 67–79.

VOGEL, W. O. P. (1981). Struktur und Organisationsprinzip im Gefäßsystem der Knochenfische. *Gegenbaurs Morph. Jb.* **127**, 772–784.

VOGEL, W. O. P. (1985). Systemic vascular anastomoses, primary and secondary vessels in fish, and the phylogeny of lymphatics. In *Cardiovascular Shunts: Phylogenetic, Ontogenetic and Clinical Aspects, Alfred Benzon Symposium 21* (ed. K. Johansen and W. Burggren), pp. 143–159. Copenhagen: Munksgaard.

VOGEL, W. O. P. AND CLAVIEZ, M. (1981). Vascular specialization in fish, but no evidence for lymphatics. *Z. Naturforsch.* **36c**, 490–492.

WALLENSTEIN, S., ZUCKER, C. L. AND FLEISS, J. L. (1980). Some statistical methods useful in circulation research. *Circulation Res.* **47**, 1–9.

WARDLE, C. S. (1971). New observations on the lymph system of the plaice *Pleuronectes platessa* and other teleosts. *J. mar. biol. Ass. U.K.* **51**, 977–990.

WOLF, K. (1963). Physiological salines for fresh-water teleosts. *Progrve Fish Cult.* **25**, 135–140.

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.

ZAR, J. H. (1984). *Biostatistical Analysis* (2nd edn). Englewood Cliffs, NJ: Prentice-Hall.