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ORIGINAL PAPER

The transition from water-breathing to air-breathing is associated with a shift in ion uptake from gills to gut: a study of two closely related erythrinid teleosts, *Hoplerythrinus unitaeniatus* and *Hoplias malabaricus*

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Abstract The evolutionary transition from water-breathing to air-breathing involved not only a change in function of the organs of respiratory gas exchange and N-waste excretion, but also in the organs of ion uptake from the environment. A combination of in vivo and in vitro techniques was used to look at the relative importance of the gills versus the gut in Na^+, Cl^-, and K^+ balance in two closely related erythrinid species: a facultative air-breather, the jeju (*Hoplerythrinus unitaeniatus*) and an obligate water-breather, the traira (*Hoplias malabaricus*). The jeju has a well-vascularized physostomous swimbladder, while that in the traira is poorly vascularized, but the gills are much larger. Both species are native to the Amazon and are common in the ion-poor, acidic blackwaters of the Rio Negro. Under fasting conditions, the traira was able to maintain positive net Na^+ and Cl^- balance in this water, and only slightly negative net K^+ balance. However, the jeju was in negative net balance for all three ions and had lower plasma Na^+ and Cl^- concentrations, despite exhibiting higher branchial Na^+,K^+ATPase and v-type H^+ATPase activities. In the intestine, activities of these same enzymes were also higher in the jeju, and in vitro measurements of net area-specific rates of Na^+, Cl^-, and K^+ absorption, as well as the overall intestinal absorption capacities for these three ions, were far greater than in the traira. When acutely exposed to disturbances in water O_2 levels (severe hypoxia ~15 % or hyperoxia ~420 % saturation), gill ionoregulation was greatly perturbed in the traira but less affected in the jeju, which could "escape" the stressor by voluntarily air-breathing. We suggest that a shift of ionoregulatory capacity from the gills to the gut may have occurred in the evolutionary transition to air-breathing in jeju, and in consequence branchial ionoregulation, while less powerful, is also less impacted by variations in water O_2 levels.

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Introduction

There is a large literature on the changes in respiration, circulation, excretion, and acid–base regulation that occurred with the evolutionary transition from water-breathing to air-breathing (e.g. Johansen 1972; Lenfant and Johansen 1972; Dejours 1975; Kramer et al. 1978; Randall et al. 1981; Ultsch 1996; Graham 1997; Sayer 2005; Glass and Rantin 2009), but far less is known about the changes which must have occurred in ionoregulation, which is another major gill function. Very simply, as the gills became smaller, less ventilated, and less perfused, this critical function had to shift to another organ, and that organ was clearly not the swimbladder (i.e. the incipient lung, Zheng et al. 2011) as it is not in contact with the water. The kidney is one organ that became more important in ionoregulation (Smith 1959; Brauner et al. 2004), but the kidney can only retain and regulate ions, not acquire them from the environment, which is a critical requirement for freshwater fish. There is also evidence that the skin became an important site of ion uptake, at least transitionally (Wilkie et al. 2007; LeBlanc et al. 2010), but in the longer term, it seems that this function must have been taken over by the gut once contact with water was lost during the evolutionary transition to life on land.

In fish, branchial ion uptake from the water has been well studied, usually in fasting fish. However, the importance of ion uptake from the food via the gut has generally been overlooked by ionoregulatory physiologists, despite evidence that trophic intake can supply 50 % or more of the normal electrolyte requirements of wild freshwater fish (Smith et al. 1989). However, that situation is changing, and more recent studies have re-inforced the quantitative importance of salt acquisition from the diet (reviewed by Wood and Bucking 2011). Recently, we have surveyed three closely related, water-breathing serrasalmid fishes with different trophic habits, all living in the ion-poor, acidic blackwaters of the Amazonian Rio Negro where even ion uptake by the gills is very challenging (Pelster et al. 2015). All three exhibited a high capacity for ion uptake via the gut in vitro, and this capacity was greater in the herbivore, which eats the most ion-poor diet, than in the omnivore or carnivore.

Building on this approach, in the present study we used a combination of in vivo and in vitro techniques, as well as activity measurements of two key ion transport enzymes (Na^+,K^+ATPase, and v-type H^+ATPase) to look at the relative importance of the gills versus the gut in net Na^+, Cl^-, and K^+ balance in two closely related species, one of which is "transitional" (a facultative air-breather), and the other of which is tied to water (an obligate water-breather). We chose two erythrinids, the jeju (*Hoplerythrinus unitaeniatus*), and the traira (*Hoplias malabaricus*), which have been studied as comparators previously (Cameron and Wood 1978; Driedzic et al. 1978; Hulbert et al. 1978a, b; Phleger and Saunders 1978; Stevens and Holeton 1978; Fernandes et al. 1994; Perry et al. 2004; Pelster et al. submitted). The gills of the two species have been studied in detail previously: these investigations have confirmed that the mass-specific branchial surface area (Cameron and Wood 1978; Fernandes et al. 1994) and the mass-specific weight of the gills (Hulbert et al. 1978b) are both twice as large in the traira (the water-breather) as those in the jeju (the air-breather). Both have a physostomous swimbladder, but in the jeju the anterior portion of the posterior chamber is heavily vascularized (red in colour) whereas in the traira, the whole swimbladder is poorly vascularized (uniformly white in colour). Facultative air-breathing using the swimbladder is well documented in the jeju (Kramer 1978; Farrell and Randall 1978; Randall et al. 1978; Stevens and Holeton 1978; Juca-Chagas 2004; Oliveira et al. 2004; Perry et al. 2004; McKenzie et al. 2007; Lopes et al. 2010), but has never been reported in the traira (Rantin et al. 1992; Val and Almeida-Val 1995; Glass and Rantin 2009). In a companion behavioural study (Pelster et al. submitted), we have confirmed this complete lack of air-breathing in the traira, even under intense hypoxia.

Our specific hypotheses were (1) that under fasting conditions, the traira would be more effective than the jeju in maintaining ion balance, because it possesses larger gills with a more extensive surface area; (2) that ion absorption capacity via the intestine (assessed in vitro) would be greater in the jeju than in the traira; (3) that during disturbances in water O_2 levels (severe hypoxia or hyperoxia), gill ionoregulary function would be perturbed to a greater extent in the traira, because jeju could "escape" the stressor by voluntarily air-breathing; and (4) that these differences in gill and gut transport capacities would be reflected in differences in the activities of key ion transport enzymes in the two tissues. A research expedition to the Anavilhanas Archipelago of the Rio Negro provided an opportunity to test these ideas in fish collected directly from the wild. To a large extent, these hypotheses were confirmed. We suggest that in the transition to air-breathing, there may have been a shift of ionoregulatory capacity from the gills to the gut, and in consequence branchial ionoregulation, while less powerful, is also less impacted by variations in water O_2 levels.

Materials and methods

Fish

All procedures were in compliance with Brazilian national and INPA animal care regulations. Experiments were performed in December 2014 on board a research vessel (the Anna Clara, from Manaus) approximately 110 km upstream from Manaus on the Rio Negro. Jeju (*H. unitaeniatus* Spix, 1829; weight = 95–165 g, fork length = 18.8–22.5 cm) and traira (*H. malabaricus* Bloch, 1794; weight = 100–275 g, fork length = 21.0–25.4 cm) were caught by INPA fishermen. The fish were held on board in large tanks served with flowing "black water" pumped directly from the Rio Negro with the following composition: $[Na^+]$ = 20–30, $[Cl^-]$ = 20–30, $[K^+]$ = 10–15, $[Ca^{2+}]$ = 10–15, $[Mg^{2+}]$ = 3–7, all in μmol L^{-1}, with pH = 4.0–4.5, dissolved organic carbon [DOC] = 8–11 mg L^{-1}, temperature = 29–35 °C (see below for analytical techniques). This water was used for all experiments, where the temperature range was held to 29–32 °C. The fish were fasted for at least 7 days prior to experimentation.

Experimental series

Series 1: basic characteristics of the two species

Basic enzymatic, plasma parameters, and respiratory parameters were compared between the two species under resting conditions. Jeju and traira were sacrificed with an overdose of MS-222 (Sigma-Aldrich, St. Louis, MO, USA), then weighed and measured (nose to caudal fin fork). A terminal 1-mL blood sample was taken by caudal puncture into a heparinized syringe (pre-rinsed with 10,000 i.u. mL^{-1} lithium heparin, Sigma-Aldrich, in Cortland saline, Wolf 1963). Blood samples were centrifuged immediately (5000 G for 1 min), and plasma was decanted and frozen in liquid nitrogen for later analyses (Na^+, K^+, Cl^-, total ammonia, urea-N, glucose, osmolality, total carbon dioxide). Gill filaments were cut from the cartilaginous branchial bars, and the entire kidney was removed. The intestine was either sampled in three sections (anterior, mid, and posterior) for the same enzymatic assays, or used to make gut sac preparations (see below). Prior to gut dissection, total intestinal length (pyloric sphincter to anus) was measured, allowing calculation of the gut length/fork length ratio. Tissue samples were rinsed with Cortland saline, blotted dry, and immediately stored in liquid nitrogen for later enzymatic assays (Na^+,K^+ATPase, v-type H^+ATPase).

For respirometry, jeju and traira were transferred to individual, light-shielded 3-L chambers fitted with air-stones and served with a constant flow of Rio Negro water, and allowed to settle for 2 h. Then water-flow and air-flow were stopped, the chambers were sealed, and changes in the water oxygen, ammonia, and urea-N concentrations were monitored over time so as to measure routine rates of O_2 consumption (MO_2), ammonia excretion (M_{Amm}), urea-N excretion ($M_{Urea\text{-}N}$), and therefore calculation of the nitrogen quotient ($[M_{Amm} + M_{Urea\text{-}N}]/MO_2$). Note that under these conditions, the fish were not allowed access to the surface, so there was no air-breathing or aquatic surface respiration (ASR). Rates were calculated in the standard fashion by factoring changes in concentration by body weight, chamber volume, and time.

Series 2: branchial flux rates with the water under normoxia, hypoxia, and hyperoxia

Net ion and N-waste flux rates with the water were compared in jeju and traira under normoxia, hypoxia, and hyperoxia. Fish were transferred to the same 3-L chambers as above. After a 2-h settling period, the water volume was set to 2.5 L. Net flux rates of Na^+, Cl^-, K^+, ammonia, and urea-N were measured in the two species under normoxia (6.0–7.6 mg L^{-1}, 83–100 % saturation), hypoxia (0.75–1.5 mg L^{-1}, 10–20 % saturation), and hyperoxia (28–35 mg L^{-1}, 370–460 % saturation), which were achieved by gassing with either air, nitrogen, or oxygen respectively. These values were selected as being realistic of normal oxygen levels seen in the Amazon basin, sometimes with fluctuations between these extremes on a diurnal basis (Kramer et al. 1978; Val and Almeida-Val 1995). Oxygen levels were monitored repetitively and gassing levels adjusted over the first 0.33 h, and then at hourly intervals thereafter to ensure that oxygen saturation stayed within the desired limits. In the normoxic control series, flux rates were measured over three or five successive 1-h intervals. In the hypoxic and hyperoxic series, fluxes were monitored initially for three 1-h normoxic periods, the chamber was flushed, and then measurements were made over four successive 1-h periods of hypoxia or hyperoxia. At each time, 10-mL water samples were taken for determination of Na^+, K^+, Cl^-, ammonia, and urea-N concentrations. Note that in these trials, the fish were allowed access to the surface, to allow the fish to exploit the possibility of air-breathing or aquatic surface respiration (ASR). Again, flux rates were calculated in the standard fashion by factoring changes in water concentration by body weight, chamber volume, and time.

Series 3: intestinal absorption rates

In vitro gut sac experiments were performed as described by Pelster et al. (2015) to quantify net Na^+, K^+, Cl^-, glucose, ammonia and fluid absorption rates from the mucosal

to the serosal side in anterior, middle and posterior gut sections of the two species. In brief, gut sections were excised, washed in ice-cold Cortland saline (Wolf 1963), and made into gut sacs of approximate 4-cm length by tying a flared polyethylene tube (Intramedic Clay-Adams PE-60; Becton–Dickinson and Company, Sparks, MD, USA) into one end and ligating the other with surgical silk suture. The sac was filled until taut with mucosal saline, and the tube was sealed. Individual initial volumes varied considerably, dependent mainly on the length of the sac. The preparation was then immersed in 15 mL of serosal saline in a 20-mL glass scintillation vial that was continuously gassed with a precision mixture (99.7 % O_2, 0.3 % CO_2) to maintain sufficient oxygen supply and normal acid -base conditions. The vials were incubated for 0.5 h in a constant temperature bath (30–32 °C) of flowing river water. The mucosal and serosal salines were identical Cortland salines, except that the mucosal solution contained 10 mmol L^{-1} NH_4Cl to mimic in vivo concentrations in chyme (e.g. Pelster et al. 2015), whereas the serosal solution lacked NH_4Cl but contained 20 mmol L^{-1} mannitol for osmotic balance. Thus the transport of water, Na^+, K^+, Cl^-, and glucose from mucosal to serosal solutions was measured under symmetrical conditions, whereas transport of ammonia was measured under asymmetrical conditions. Initial and final samples of mucosal and serosal saline were frozen for analysis. The weight change of the preparation over 0.5 h was used to calculate fluid absorption rate, and the measured changes in saline composition over this same period, together with gut sac fluid volume changes, were used to calculate net Na^+, K^+, Cl^-, glucose, and ammonia absorption rates. Flux rates were expressed per unit surface area (cm^2), which was measured at the end of the experiment by cutting the gut sac open and tracing its perimeter onto 0.5-mm graph paper (Grosell and Jensen 1999). This technique measured the gross surface area as there were no obvious major folds in the gut tissue of either species, but did not measure the area contributed by internal subdivision of the surface into villi and microvilli. These tracings also allowed calculation of the area of the gut section per unit length (cm^2 cm^{-1}) of the gut which was then multiplied by the total length (cm) to yield the total area (cm^2) of each gut section. By addition of the three sections, the total gut surface area per unit body mass (cm^2 kg^{-1}) could be computed. Specific calculation details are given by Pelster et al. (2015).

Analytical procedures

Water oxygen concentration was recorded with a portable oxygen electrode and meter (WTW Oxi325 Oximeter, Weilheim, Germany). Na^+ and K^+ concentrations in water, plasma, and gut sac salines were measured with a 910 Digital Flame Photometer (Instrumentação Analítica São Paulo, SP, Brazil). Cl^- concentrations in these same solutions were measured by colorimetric assay (Zall et al. 1956). Water Ca^{2+} and Mg^{2+} concentrations were measured by atomic absorption spectrophotometry using a SpectrAA 220FS (Varian Canada, Missisauga, ON, Canada). DOC concentrations were quantified by combustion using a Shimadzu TOC-$V_{CPH/CPN}$ total organic carbon analyzer (Shimadzu Corporation, Kyoto, Japan) on water samples which had been first passed through a 0.45 µm filter (Acrodisk Supor Membrane, Pall Life Sciences, Port Washington, NY, USA). Water pH was monitored by a Symphony SP70P meter and probe (VWR, Radner, PA, USA). Plasma osmolality was measured with a vapor pressure osmometer (Advanced Micro Osmometer, Model 3300, Advanced Instruments, Norwood, MA, USA), plasma total carbon dioxide concentration with a Corning 965 Total CO_2 Analyser (Ciba-Corning, Halstead, Essex, UK). Total ammonia and urea concentrations in water were measured by the colorimetric assay of Verdouw et al. (1978) and Rahmatullah and Boyde (1980), respectively. The latter was also used for urea assays on plasma, but ammonia concentrations in plasma and gut sac salines were determined enzymatically using a Raichem commercial assay (Cliniqa Corporation, San Marcos, CA, USA) so as to avoid protein-based interference in the Verdouw et al. (1978) assay. Glucose concentrations in plasma and gut sac salines were measured using the Infinity™ Glucose Hexokinase Liquid Stable Reagent from Thermo Fisher Scientific Inc. (Burlington, ON, Canada).

Na^+,K^+ATPase (NKA) and v-type H^+ATPase were measured according Kültz and Somero (1995). The assay is based on the inhibition of the NKA activity by ouabain (2 mmol L^{-1}), and of v-type H^+ATPase by N-ethylmaleimide (NEM, 2 mmol L^{-1}) in a reaction mixture (fresh made) containing 30 mmol L^{-1} imidazole, 45 mmol L^{-1} NaCl, 15 mmol L^{-1} KCl, 3 mmol L^{-1} $MgCl_2$, 0.4 mmol L^{-1} KCN, 1 mmol L^{-1} Na_2ATP, 0.2 mmol L^{-1} NADH, 0.1 mmol L^{-1} fructose 1,6-biphosphate, 2 mmol L^{-1} phosphoenolpyruvate (PEP), 3 international units (IU) mL^{-1} pyruvate kinase, and 2 IU mL^{-1} lactate dehydrogenase (LDH). A reaction mixture without any inhibitor was used to measure the total ATPase activity. Tissues samples were homogenized (1:10 for gills and intestine portions and 1:40 for kidney, w:v) in a buffer (pH 7.5 containing 150 mmol L^{-1} sucrose, 50 mmol L^{-1} imidazole, 10 mmol L^{-1} EDTA, and 2.5 mmol L^{-1} deoxycholic acid, and then centrifuged at 2000*g* for 5 min at 4 °C. All the assays were performed at 25 °C by combining 200 µL of the reaction mixture (with ouabain, or with NEM or without inhibitors) and 5 µL of the homogenate. The change in the absorbance was read over 10 min at 340 nm. NKA and v-type H^+ATPase activities were calculated as the difference between total activity and activities with ouabain and NEM inhibitors

respectively. Total protein was measured according Bradford (1976) using bovine serum albumin (BSA) as a standard at 595 nm. NKA and v-type H^+ATPase activities were expressed as μmol ATP mg protein^{-1} h^{-1}.

Statistics

Data have been expressed as means ± 1 SEM (N = number of animals, or number of individual gut sac or enzyme preparations from different animals). Student's two-tailed t test or a Mann–Whitney ranked sum non-parametric test, as appropriate, was used for comparison of basic parameters between jeju and traira. For statistical analysis of net flux rates of whole animals over time, two-way ANOVA (species, time) was used, followed by the Holm-Sidak multiple comparison procedure to compare back to control normoxic measurements. For the comparison of flux rates of the different gut sections, as well as for their enzymatic activities, two-way ANOVA (species, sections), followed by the Holm-Sidak multiple comparison test, was employed. In rare cases the normality test or equal variance test failed using the original data. In these situations, the data were log transformed prior to statistical analysis. Statistical analyses were performed using SigmaStat 12.0 at a significance level of $P < 0.05$.

Results

Series 1: basic characteristics of the two species

The two species were of similar weight though the traira were longer (Table 1). The gut length to fork length ratio was the same in the two species, but both the condition factor and the total gut area per unit body mass were significantly greater in the jeju. Under resting normoxic conditions without access to air, routine MO_2 was about 50 % higher in jeju than in traira (Table 1). Routine M_{Amm} did not differ between the two species, but routine $M_{Urea\text{-}N}$ was twice as high in the jeju. However, the nitrogen quotient (NQ) did not differ significantly.

Plasma Na^+ and Cl^- concentrations were significantly lower in the jeju than in the traira, but there were no differences in plasma K^+ or urea (Table 2). Plasma total ammonia and osmolality were not significantly different between the two species, but note the low N number for these measurements in jeju due to sample loss. Plasma total CO_2 concentration was twice as high in the facultatively air-breathing jeju, and plasma glucose levels were similarly higher. In all tissues (intestine, gills, gut), v-type H^+ATPase activities were greater than Na^+,K^+ATPase activities (Fig. 1; Table 3). Intestinal activities of both enzymes were significantly higher overall in the jeju than the traira by two-way ANOVA, in agreement with our original hypotheses, with significant individual differences in the posterior intestine for Na^+,K^+ATPase (Fig. 1a), and in all three sections for v-type H^+ATPase (Fig. 1b). There were no differences among sections for either species. In the kidney, there were no differences between species for Na^+,K^+ATPase, but v-type H^+ATPase activity was about 50 % higher in the traira. However, in the gills, the activities of both enzymes were unexpectedly higher in the jeju by 3.5-fold for Na^+,K^+ATPase and 1.6-fold for v-type H^+ATPase (Table 3), contrary to our initial ideas.

Table 1 Basic morphological and respiratory parameters in jeju (*Hoplerythrinus unitaeniatus*) and traira (*Hoplias malabaricus*) under normoxic control conditions in Series 1

	Jeju	Traira
Body mass (kg)	0.12 ± 0.01 (16)	0.12 ± 0.01 (32)
Fork length (cm)	20.1 ± 0.7 (5)	25.4 ± 1.2 (8)*
Gut length (cm)	14.9 ± 0.6 (5)	18.0 ± 1.8 (8)
Gut length/fork length ratio	0.74 ± 0.04 (5)	0.70 ± 0.04 (8)
Condition factor	1.44 ± 0.07 (5)	1.05 ± 0.02 (8)*
Total gut area/body mass (cm^2 kg^{-1})	82.7 ± 9.0 (5)	56.6 ± 2.9 (6)*
MO_2 (mmol kg^{-1} h^{-1})	5.6 ± 0.6 (7)	3.8 ± 0.2 (8)*
Ammonia excretion (μmol kg^{-1} h^{-1})	785 ± 62 (13)	630 ± 58 (8)
Urea excretion (μmol kg^{-1} h^{-1})	145 ± 21 (7)	65.2 ± 12.0 (8)*
NQ ratio	0.17 ± 0.01 (7)	0.19 ± 0.02 (8)

* Significant difference ($P < 0.05$) between the two species. Means ± 1 SEM (N)

Series 2: branchial flux rates with the water under normoxia, hypoxia, and hyperoxia

In accordance with the lower plasma Na^+ and Cl^- concentrations, and in agreement with one of our original hypotheses (see "Introduction"), there were striking differences in the net flux rates of these two electrolytes with the external water under normoxic conditions (Fig. 2a, b). Traira were in net positive balance for both Na^+ and Cl^- (approximately + 100 μmol kg^{-1} h^{-1}) throughout 5 h of normoxia, whereas jeju were in net negative balance for Na^+ (approximately −150 μmol kg^{-1} h^{-1}), while net Cl^- balance started close to zero but became negative over time. The data from the 3- and 5-h experiments have been combined in Fig. 2 because there were no significant time-dependent differences within either species, whereas the overall differences between the species for both ions were significant (two-way ANOVA), with many of the individual differences also being significant at comparable time points. Net K^+ balance was negative in both species, but absolute net flux rates were about three-fold greater (−90 versus

Table 2 Basic plasma parameters in jeju (*Hoplerythrinus unitaeniatus*) and traira (*Hoplias malabaricus*) under normoxic control conditions in Series 1

	Jeju	Traira
Plasma Na^+ (mmol L^{-1})	131.4 ± 7.5 (5)	147.8 ± 1.9 (20)*
Plasma Cl^- (mmol L^{-1})	93.6 ± 6.5 (5)	111.2 ± 2.1 (20)*
Plasma K^+ (mmol L^{-1})	4.4 ± 1.2 (5)	4.8 ± 0.3 (20)
Plasma total CO_2 (mmol L^{-1})	13.2 ± 0.8 (5)	6.1 ± 0.4 (20)*
Plasma osmolality (mmol L^{-1})	252.2 ± 17.4 (3)	265.5 ± 2.4 (18)
Plasma total ammonia (mmol L^{-1})	0.16 ± 0.03 (3)	0.08 ± 0.02 (13)
Plasma urea (mmol L^{-1})	4.10 ± 0.85 (5)	3.99 ± 0.22 (20)
Plasma glucose (mmol L^{-1})	6.2 ± 1.1 (5)	3.2 ± 0.1 (15)*

* Significant difference ($P < 0.05$) between the two species. Means ± 1 SEM (*N*)

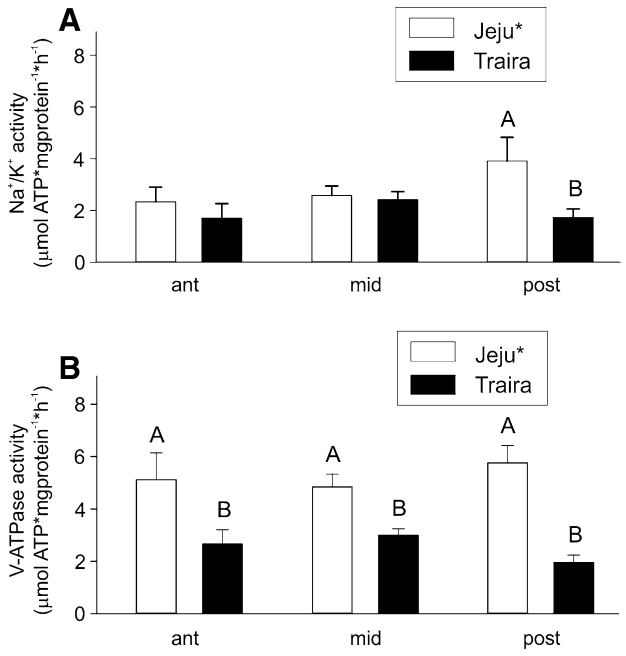

Fig. 1 A comparison of (**a**) Na^+,K^+ATPase and (**b**) v-type H^+ATPase activities in the anterior, mid, and posterior sections of the intestine in jeju (*open bars* $N = 5$) versus traira (*black bars* $N = 19$) in Series 1. Means + 1 SEM. Within each section, *bars* marked by *different letters* indicate significant differences ($P < 0.05$) between the two species. Within each species, there were no significant differences among sections. The *asterisks* on "Jeju" indicate overall species differences by two-way ANOVA

−30 µmol kg^{-1} h^{-1}) in the jeju, a difference that was significant overall and at most time points (Fig. 2c). Again there were no time-dependent differences. Thus under normoxic conditions with access to air, fasted traira can use their gills to maintain positive Na^+ and Cl^- balance, and an only slightly negative K^+ balance. In contrast, fasted jeju cannot do this.

Table 3 Na^+,K^+ ATPase and v-type H^+-ATPase activity (µmol ATP^{-1} mg $protein^{-1}$ h^{-1}) in gills and kidney of jeju (*Hoplerythrinus unitaeniatus*) and traíra (*Hoplias malabaricus*) in Series 1

	Jeju ($N = 5$)	Traira ($N = 19$)
Gills		
Na^+,K^+ATPase	2.84 ± 0.14	0.80 ± 0.10*
v-type H^+ATPase	5.08 ± 0.17	3.16 ± 0.16*
Kidney		
Na^+,K^+ATPase	1.60 ± 0.13	1.32 ± 0.08
v-type H^+ATPase	1.72 ± 0.04	2.58 ± 0.11*

Means ± 1 SEM (N)

* Significant difference ($P < 0.05$) between the two species

As there were no significant time-dependent differences under normoxia (Fig. 2), data from the three pre-exposure normoxic control periods were averaged for comparison to the 4 h of hypoxia (Fig. 3) or hyperoxia (Fig. 4). Overall, the results demonstrated that net ion balance at the gills of traira was substantially disturbed by large alterations in water O_2 tensions, while these had little effect in jeju that had the option to breathe air during these treatments, in accordance with our original hypotheses.

In jeju, exposure to approximately 15 % O_2 saturation in the water, with access to air allowed, had no effect on net Na^+, Cl^-, or K^+ balance (Fig. 3a–c); values remained highly negative at levels comparable to the normoxic control. However, in traira, the same hypoxia treatment significantly disturbed net Na^+ and K^+ balance, both of which became negative, and significantly different from the normoxic control values at 2 h for Na^+ (Fig. 3a), and at 1, 2, and 3 h for K^+ (Fig. 3c). Net Cl^- balance changed from slightly positive to negative, but none of the hourly values were significantly different from the control (Fig. 3b). However, two-way ANOVAs revealed a significant overall effect of hypoxia on the fluxes of all three ions in traira, but not in jeju. There were also overall significant species-dependent differences during both normoxia and hypoxia.

As with hypoxia, exposure to approximately 420 % O_2 saturation in the water, with access to air allowed, again had no significant effect on the hourly net flux rates of Na^+, Cl^-, or K^+ in jeju (Fig. 4a–c). However, Cl^- balance became consistently negative, and a two-way ANOVA revealed a significant overall effect of hypoxia on net Cl^- fluxes, but not on the fluxes of the other two ions in jeju. In traira, the same hyperoxia treatment disturbed hourly net Cl^- balance which became significantly negative at 2 and 3 h (Fig. 4b). Net Na^+ balance also became transiently negative at 1 and 2 h, but none of the hourly values were significantly different from the control (Fig. 4a). Net K^+ balance was not affected (Fig. 4c). A two-way ANOVA revealed a significant overall effect of hyperoxia on net

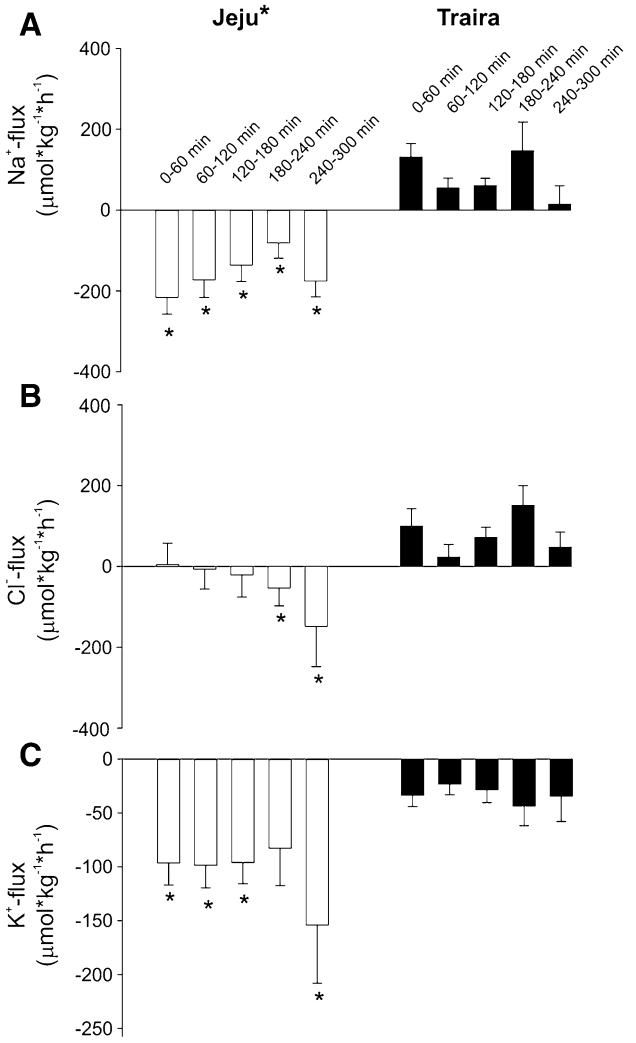

Fig. 2 A comparison under normoxia in jeju (*open bars*) versus traira (*black bars*) of the net flux rates with the water of (**a**) Na^+, (**b**) Cl^-, and (**c**) K^+ in Series 2. Fluxes were measured over successive 1-h periods under normoxic conditions in which the fish were allowed access to air. For jeju, $N = 13$ for the first, second, and third hours and $N = 8$ for the fourth and fifth hours. For traira, $N = 26$ for the first, second, and third hours and $N = 7$ for the fourth and fifth hours. Positive values represent net gains by the fish, negative values indicate net losses by the fish. Means + 1 SEM. *Asterisks* indicate significant differences ($P < 0.05$) between the two species at the same time point. Within each species, there were no significant differences among times. Two-way ANOVA indicated significant overall species differences for all three ions, as indicated by the asterisk on "Jeju"

Cl^- balance, but not on the fluxes of the other two ions. The overall species-dependent differences between jeju and traira were again significant for all three ions during both normoxia and hyperoxia.

M_{Amm} and $M_{Urea\text{-}N}$ were also measured during the three treatments, and as there were no significant differences over time, have been averaged in Fig. 5. In these experiments, where access to air was allowed, both M_{Amm} (Fig. 5a) and $M_{Urea\text{-}N}$ (Fig. 5b) were significantly greater during normoxia in jeju than in traira, though the relative difference was far greater for $M_{Urea\text{-}N}$. This species difference for $M_{Urea\text{-}N}$ was maintained during hyperoxia, but not during hypoxia (Fig. 5b). Exposure to hypoxia significantly depressed M_{Amm} in both species (Fig. 5a), while exposure to hyperoxia was without effect on either M_{Amm} or $M_{Urea\text{-}N}$. Hypoxia also decreased $M_{Urea\text{-}N}$ in jeju. Overall, there was a significant species effect for $M_{Urea\text{-}N}$ but not for M_{Amm} by a two-way ANOVA.

Series 3: intestinal absorption rates

In vitro gut sac experiments demonstrated substantially higher absorption rates of ions and nutrients in the intestine of the facultative air-breather (jeju) than in the water-breather (traira), in agreement with our initial hypotheses.

Area-specific rates of net Na^+, Cl^-, and K^+ absorption were significantly greater by fivefold (Fig. 6a), twofold (Fig. 6b), and fourfold (Fig. 6c) respectively in the anterior intestine of jeju relative to traira. There were smaller non-significant differences in the mid and posterior intestine. Two-way ANOVAs confirmed that there were significant overall species-dependent differences for all three ions, but no significant differences amongst segments. Despite the differences in ion flux rates, there were no significant differences between species in fluid absorption rates (Fig. 6d).

Area-specific rates were converted to total rates per unit body mass by taking total gut area into account (Fig. 7). Two-way ANOVAs again demonstrated that there were significant overall species-dependent differences for all three ions, and in addition, some segment-specific differences for K^+ (Fig. 7c) but not for the other two ions. Total ion transport capacities were much greater in the intestine of the jeju, with significant sevenfold differences for Na^+ (Fig. 7a) and K^+ (Fig. 7c) and a twofold difference for Cl^- (Fig. 7b). In addition to exhibiting the greatest area-specific differences (Fig. 6), the anterior sections generally had the greatest total areas, so their contributions to total transport capacities were greatest. This combination also resulted in a significantly higher fluid transport capacity in the anterior intestine of the jeju relative to traira, but not in the other two sections or overall (Fig. 7d).

Area-specific rates of net ammonia absorption did not differ between species or among intestinal segments (Fig. 8a). For net glucose absorption, there were again no differences in area-specific rates between species, but there were significantly higher rates in the anterior versus the posterior intestine in jeju only (Fig. 8b). However, when converted to total rates per unit body mass, total intestinal absorption capacities were significantly higher for both ammonia and glucose in jeju versus traira, with greatest contributions by significant differences in the capacities

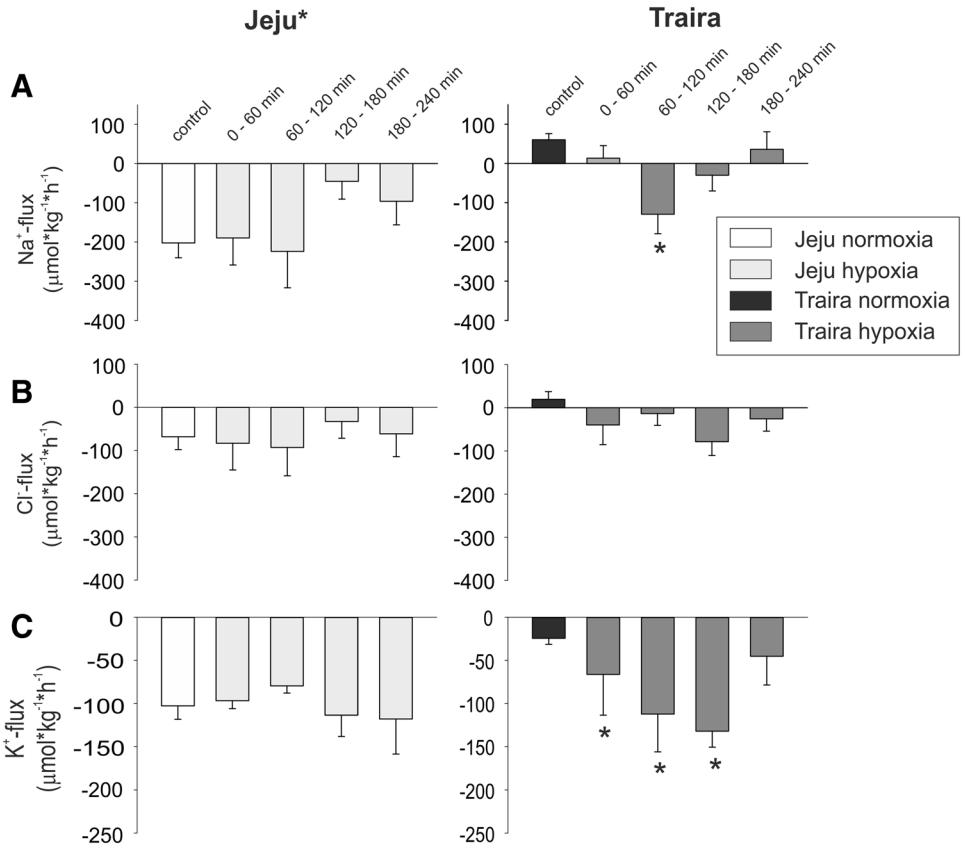

Fig. 3 A comparison of the responses to severe waterborne hypoxia of jeju versus traira of the net flux rates with the water of (**a**) Na^+, (**b**) Cl^-, and (**c**) K^+ in Series 2. The three 1-h normoxic control periods have been averaged (*open bars* for jeju, *black bars* for traira). Under hypoxia (approximately 15 % saturation), fluxes were measured over successive 1-h periods (*light grey bars* for jeju, *dark grey bars* for traira) during which the fish were allowed access to air. For jeju, $N = 5$, for traira $N = 12$. Positive values represent net gains by the fish, negative values indicate net losses by the fish. Means + 1 SEM. *Asterisks* indicate significant differences ($P < 0.05$) from the control value. Two-way ANOVA indicated a significant overall effect of hypoxia on the fluxes of all three ions in traira, but not in jeju. There were also overall significant differences between the species during both normoxia and hypoxia, as indicated by the *asterisk* on "Jeju"

of anterior intestine (Fig. 9a, b). There were also segment-specific differences (anterior = highest, posterior = lowest) for ammonia absorption capacity in the jeju, and for glucose absorption capacity in both species.

Discussion

Overview

By comparing two closely related erythrinid species, we have provided evidence for the idea that there may have been a transition in the relative importance of the gut versus the gills for ion acquisition accompanying the evolutionary transition from water-breathing to air-breathing. In its native ion-poor water under normoxic conditions, the traira, an obligatory water-breather with a non-vascularized swimbladder but large gills, was able to maintain generally positive ion balance without feeding. In contrast, the jeju, a facultative air-breather with a well-vascularized swimbladder but smaller gills, exhibited negative balance under the same conditions, suggesting that ion uptake from the diet was essential. Furthermore, the capacity of the gut for ion acquisition, as assessed in vitro, was much greater in the jeju. When acutely exposed to severe hypoxia or hyperoxia, the water-breathing traira suffered disturbance of branchial ion balance, whereas the facultatively air-breathing jeju was able to largely avoid this problem. We suggest that a shift of ionoregulatory capacity from the gills to the gut may have occurred in the evolutionary transition to air-breathing, and in consequence branchial ionoregulation, while less powerful, is also less impacted by variations in water O_2 levels in air-breathing fish.

Branchial ionoregulation

Our conclusion that under normoxia, the gills of the jeju are less effective in net ion acquisition than those of the traira, is based on the inherent assumption that ion fluxes with the water (Fig. 2) largely reflect ion fluxes at the gills in these fasted fish. Ion losses through the kidney were not measured in the present study. However, Cameron and Wood (1978) directly cannulated the urinary papilla to collect excreted urine in these same two species, and reported that under normoxia, urinary Na^+ and Cl^- efflux rates were significantly higher in the jeju, but amounted to only 10 % of the unidirectional Na^+ efflux rates at the gills in the jeju, and only 4 % in the traira. Thus the kidney appears to play at most a small role in the difference between the species. The higher v-type H^+ATPase activity in the kidney of the traira (Table 3) may contribute to this difference by greater ion scavenging from the urine.

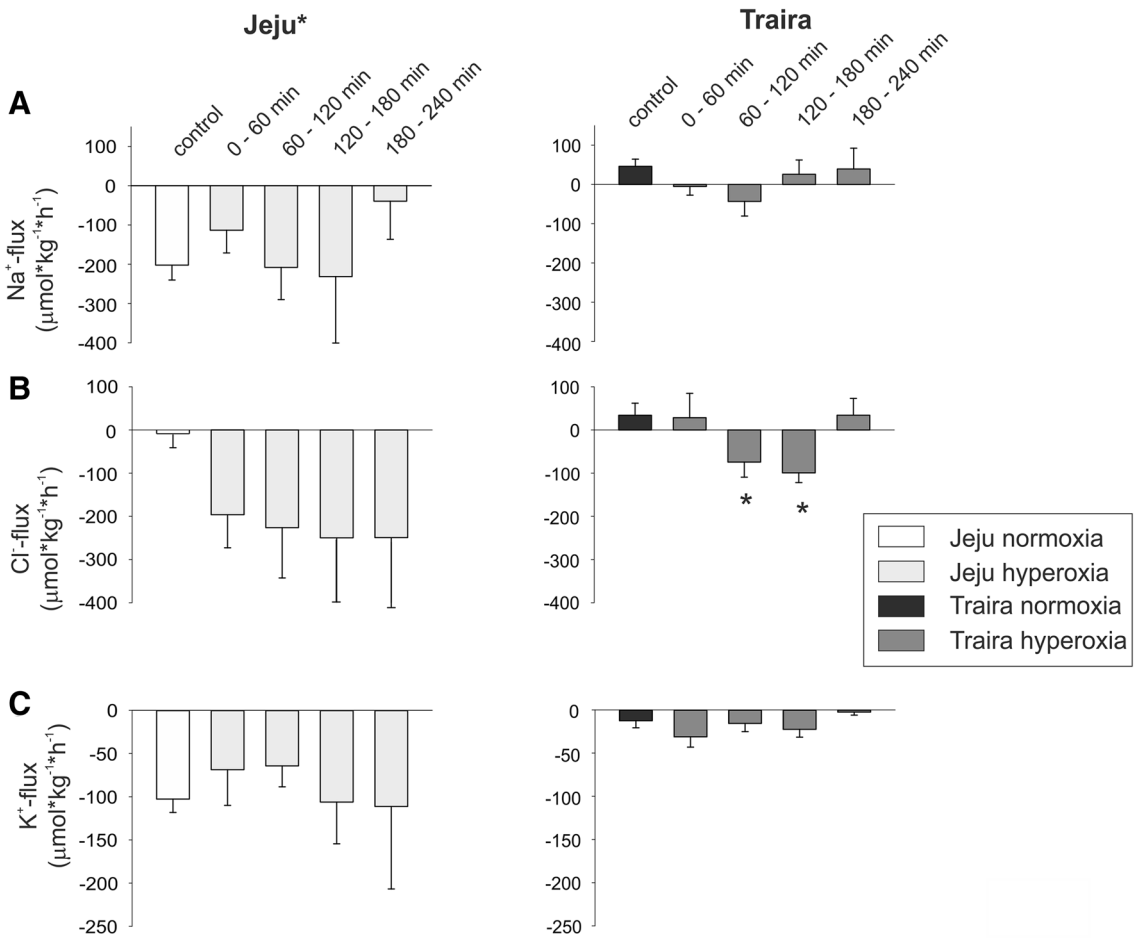

Fig. 4 A comparison of the responses to waterborne hyperoxia of jeju versus traira of the net flux rates with the water of (**a**) Na^+, (**b**) Cl^-, and (**c**) K^+ in Series 2. The three 1-h control periods have been averaged (*open bars* for jeju, *black bars* for traira). Under hyperoxia (approximately 420 % saturation), fluxes were measured over successive 1-h periods (*light grey bars* for jeju, *dark grey bars* for traira) during which the fish were allowed access to air. For jeju, $N = 6$, for traira, $N = 12$. Positive values represent net gains by the fish, negative values indicate net losses by the fish. Means + 1 SEM. *Asterisks* indicate significant differences ($P < 0.05$) from the control value. Two-way ANOVA indicated a significant overall effect of hyperoxia on the net Cl^- flux in both species, but not on the fluxes of the other two ions. There were also overall significant differences between the species during both normoxia and hyperoxia, as indicated by the *asterisk* on "Jeju"

Unidirectional Na^+ and Cl^- efflux rates under normoxia (Fig. 2) must have exceeded unidirectional influx rates at the gills in the jeju (and vice versa in the traira), but it is unclear whether the inter-species differences were due to higher active branchial influx rates, lower passive branchial efflux rates, or both, in the traira versus the jeju. Cameron and Wood (1978) reported no differences in either branchial Na^+ influx or efflux rates between the two species, but did not control feeding and worked on cannulated animals held in water containing about tenfold higher ion levels, so direct comparisons cannot be made. Based on the approximately 50 % smaller measured gill surface area and size in the jeju (Hulbert et al. 1978b; Cameron and Wood 1978; Fernandes et al. 1994), and the qualitative conclusion of Hulbert et al. (1978a) that mitochondrial-rich cells are less abundant in the gills of the jeju, it seems likely that active unidirectional Na^+ and Cl^- influx rates would also be lower in this species. However, we were surprised to find that branchial Na^+,K^+ATPase activities were 3.5-fold higher, and branchial v-type H^+ATPase activities were 1.6-fold higher in the jeju (both expressed per mg protein; Table 3). Hulbert et al. (1978a) used a different Na^+,K^+ATPase assay and expressed activities per g gill tissue, but reached the same qualitative conclusion—higher activity in the jeju gills by 1.33 fold. Thus to some extent, the jeju compensates for its smaller gill size by exhibiting greater activity levels of these two key ion transport enzymes per unit of tissue or protein. Net K^+ loss rates have been interpreted as indices of branchial diffusive permeability (Laurén and McDonald 1985; Wood et al. 2009; Iftikar et al. 2010), so the much more negative K^+ fluxes in the jeju under normoxia

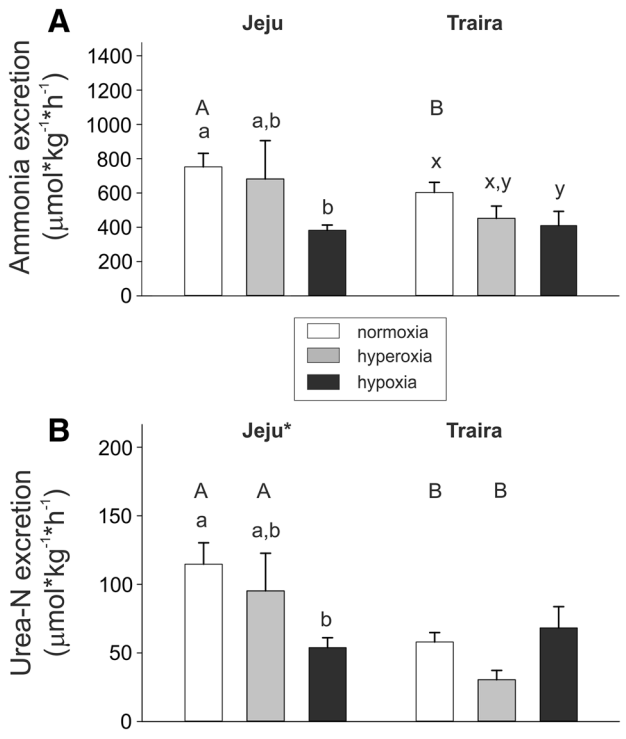

Fig. 5 A comparison of (**a**) ammonia excretion rates and (**b**) urea-N excretion rates between jeju and traira under normoxia (*open bars*), hyperoxia (*grey bars*), and hypoxia (*black bars*) in Series 2. The fish were allowed access to air under all three conditions. For jeju, $N = 11$ during normoxia, $N = 5$ during hypoxia, and $N = 6$ during hyperoxia. For traira, $N = 24$ during normoxia, $N = 12$ during hypoxia, and $N = 12$ during hyperoxia. Means + 1 SEM. *Upper case letters* indicate significant differences ($P < 0.05$) between the two species under the same condition; *lower case letters* indicate significant differences between conditions within each species. *Bars* sharing the *same letter* are not significantly different. The *asterisk* on "Jeju" indicates an overall species difference by two-way ANOVA

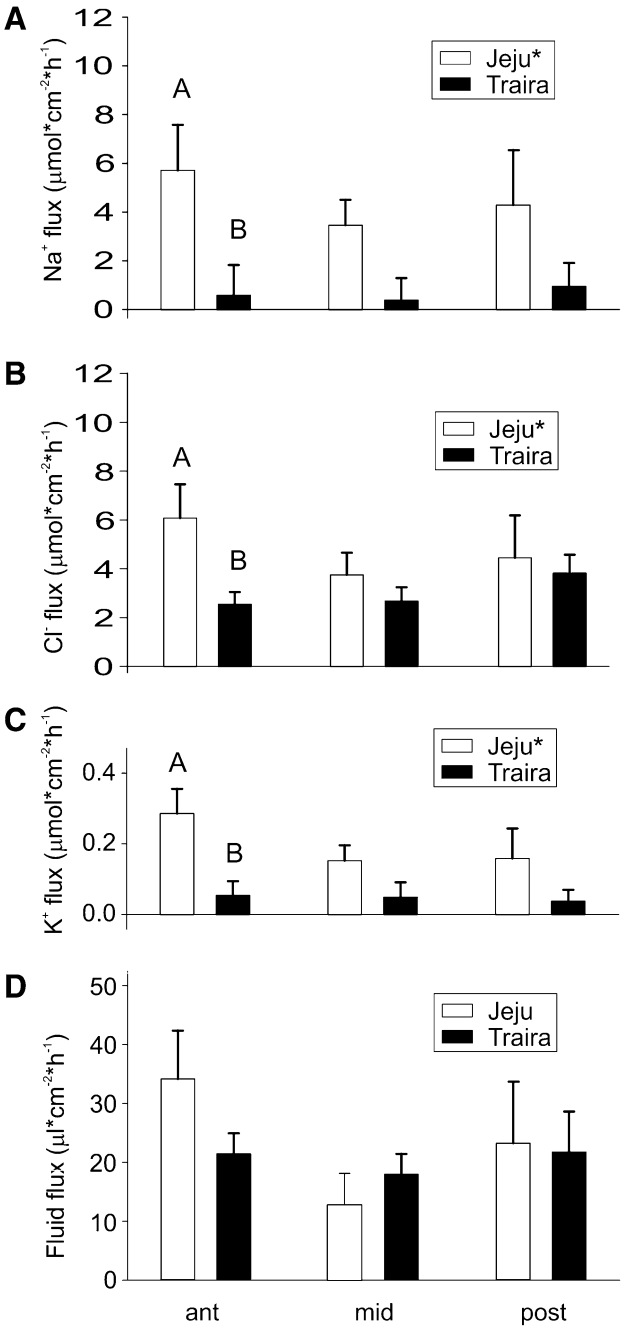

Fig. 6 A comparison between jeju (*open bars* $N = 5$) and traira (*black bars* $N = 8$) of the net absorption rates of (**a**) Na^+, (**b**) Cl^-, (**c**) K^+, and (**d**) fluid in the anterior, mid, and posterior sections of the intestine in Series 3. Rates were measured in gut sac preparations in vitro and are expressed per unit of intestinal surface area (cm^2). Means + 1 SEM. *Upper case letters* indicate significant differences ($P < 0.05$) between the two species for the same section. There were no significant differences among sections within each species. *Bars* sharing the *same letter* are not significantly different. The *asterisks* on "Jeju" indicate overall species differences by two-way ANOVA

(Fig. 2) point to a higher baseline permeability (i.e. higher diffusive efflux rates) of the gills. However, the fact that the net K^+ fluxes of the traira became much more negative during hypoxia, whereas those of the jeju did not (Fig. 3), is likely more a function of increased gill permeability in the traira (i.e. the osmorespiratory compromise) than it is of gill size which would have been unchanged.

Indeed, exposure to severe hypoxia (~15 % O_2 saturation) in the water resulted in a changeover to negative net Na^+, Cl^-, and K^+ balances in the traira, all classic symptoms of the traditional osmorespiratory compromise (Fig. 3): ions are lost as effective branchial permeability is increased in an attempt to acquire more O_2 from the environment (e.g. Randall et al. 1972; Iftikar et al. 2010; Robertson et al. 2015). Jeju were able to avoid this effect (Fig. 3) by switching to an increased reliance on air-breathing. In our companion study (Pelster et al. submitted), the same approximate level of waterborne hypoxia increased air-breathing frequency by about sixfold, and increased time spent at the surface by about tenfold, in qualitative agreement with many previous studies (Kramer 1978; Farrell and Randall 1978; Randall

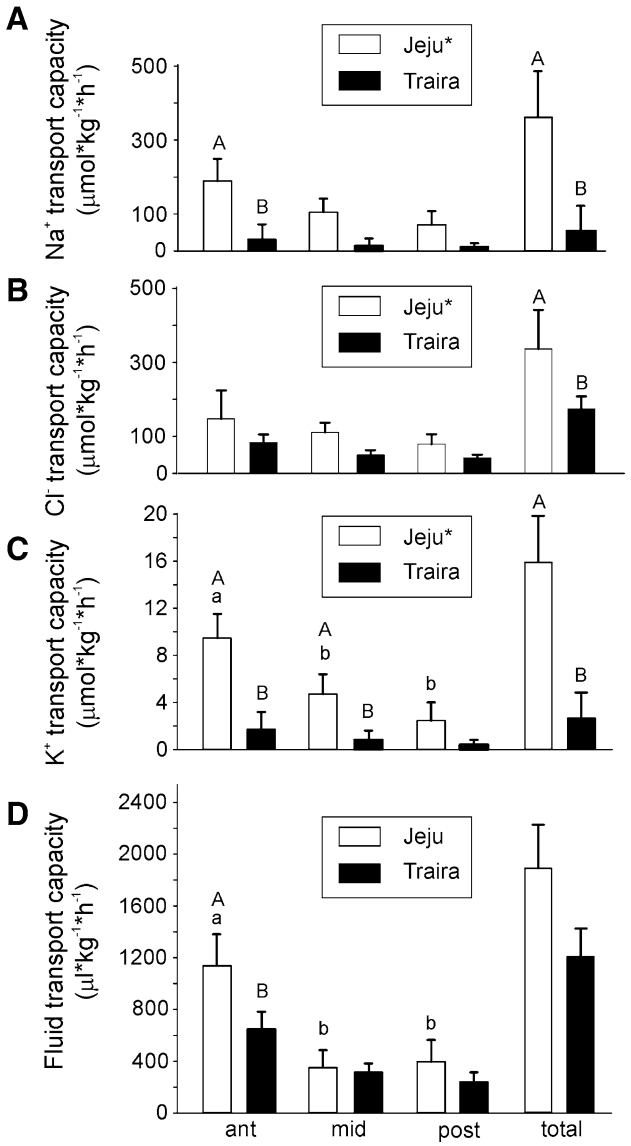

Fig. 7 A comparison between jeju (*open bars* $N = 5$) and traira (*black bars* $N = 8$) of the net absorption capacities for (**a**) Na^+, (**b**) Cl^-, (**c**) K^+, and (**d**) fluid in the anterior, mid, and posterior sections of the intestine in Series 3. The far *right-hand pair* of bars in each panel represents the total capacities. Capacities were calculated from flux rate data (Fig. 6) and morphometric measurements of the gut, and are expressed per kg of body mass. Means + 1 SEM. *Upper case letters* indicate significant differences ($P < 0.05$) between the two species for the same section; *lower case letters* indicate significant differences among sections within each species. *Bars* sharing the *same letter* are not significantly different. The *asterisks* on "Jeju" indicate overall species differences by two-way ANOVA

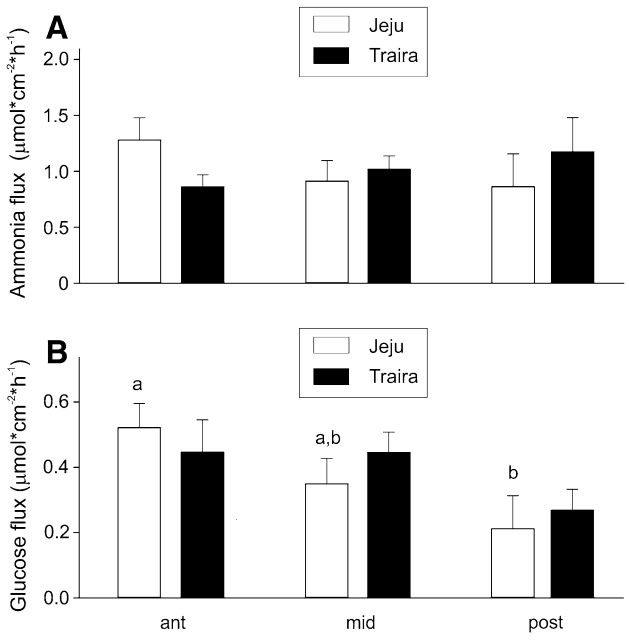

Fig. 8 A comparison between jeju (*open bars* $N = 5$) and traira (*black bars* $N = 8$) of the net absorption rates of (**a**) ammonia and (**b**) glucose in the anterior, mid, and posterior sections of the intestine in Series 3. Rates were measured in gut sac preparations in vitro and are expressed per unit of intestinal surface area (cm^2). Means + 1 SEM. *Lower case letters* indicate significant differences ($P < 0.05$) among sections within the same species. There were no significant differences between the two species for the same section. *Bars* sharing the *same letter* are not significantly different. There were no overall species differences by two-way ANOVA

et al. 1978; Stevens and Holeton 1978; Juca-Chagas 2004; Oliveira et al. 2004; Perry et al. 2004; McKenzie et al. 2007; Lopes et al. 2010). Quantitatively, these reports vary in the extent of reliance on air-breathing at various levels of water PO_2, but it is clear that for the jeju under severe hypoxia, air-breathing is critical to survival, but a matter of choice when under normoxia. Certainly, when denied access to air under normoxia, jeju were able to maintain routine MO_2 at a rate about 50 % higher than in traira, while urea excretion was about twofold higher (Table 1). While this may seem surprising given their smaller gills, it must be appreciated that the branchial surface area of the jeju is actually the same as in the similarly sized rainbow trout, a highly aerobic teleost (Cameron and Wood 1978). During hypoxia, this metabolic rate was likely compromised because both ammonia and urea excretion were substantially reduced in the jeju, while only ammonia excretion fell in the traira (Fig. 5).

Exposure to waterborne hyperoxia (~420 % O_2 saturation) disturbed branchial Cl^- balance in both species, with more pronounced effects in the traira than in the jeju (Fig. 4). Interestingly, the jeju tended to escape hyperoxia by a greater reliance on air-breathing, as occurred in hypoxia (Pelster et al. submitted), although the increases in air-breathing frequency and time spent at the surface were both modest (2 to 4-fold). Increased air-breathing in response to waterborne hyperoxia has not been reported before in the jeju, but has been seen in at least one other facultatively air-breathing teleost, the Magadi tilapia (*Alcalicus grahami*) (Johannsson et al. 2014). Both investigations attributed this behaviour to

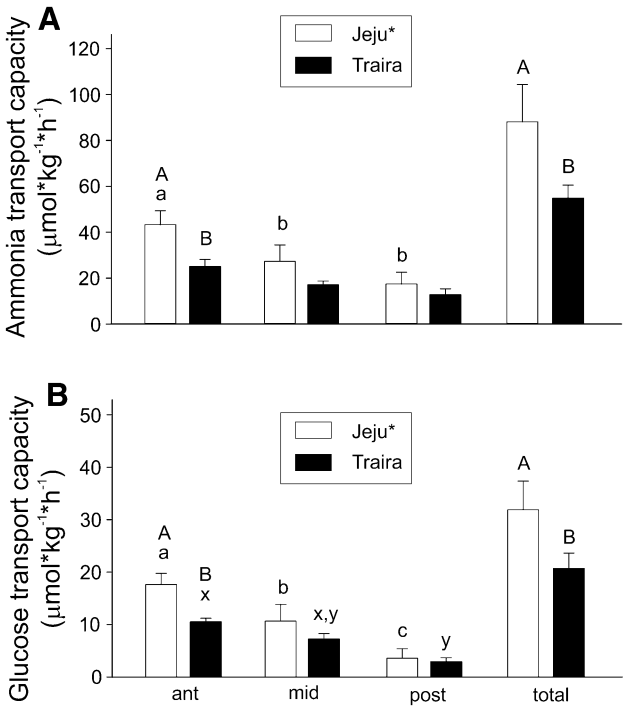

Fig. 9 A comparison between jeju (*open bars* $N = 5$) and traira (*black bars* $N = 8$) of the net absorption capacities for (**a**) ammonia and (**b**) glucose in the anterior, mid, and posterior sections of the intestine in Series 3. The far *right-hand pair* of bars in each panel represents the total capacities. Capacities were calculated from flux rate data (Fig. 8) and morphometric measurements of the gut, and are expressed per kg of body mass. Means + 1 SEM. *Upper case letters* indicate significant differences ($P < 0.05$) between the two species for the same section; *lower case letters* indicate significant differences among sections within each species. *Bars* sharing the *same letter* are not significantly different. The *asterisks* on "Jeju" indicate overall species differences by two-way ANOVA

a strategy for limiting the exposure of the tissues to reactive oxygen species (ROS), and therefore for minimizing oxidative damage (Lushchak and Bagnyukova 2006). Possibly, the elevated branchial Cl^- loss rates in both species are symptomatic of such oxidative damage to the gills, but more likely they reflect acid–base compensation. It is well-established that water-breathing fish respond to waterborne hyperoxia by retaining CO_2, and that the accompanying respiratory acidosis is compensated by HCO_3^- retention achieved by slowing the rate of Cl^- influx *versus* HCO_3^- efflux exchange at the gills (e.g. Wood et al. 1984; Truchot 1987). The same probably also occurs in facultative air-breathers when the water becomes hyperoxic, though we are aware of no direct evidence on this point. Thus net Cl^- balance becomes negative. Plasma HCO_3^- levels were not measured in the present study, but plasma total CO_2 concentration (mainly HCO_3^-) under normoxia was greater in the jeju than in the traira, as would be expected in a partial air-breather with a higher blood PCO_2 (Dejours 1988; Truchot 1987; Ultsch 1996).

Intestinal ionoregulation

In accordance with predictions, the jeju was much more effective than the traira in the uptake of ions via the intestine. This difference was particularly pronounced for net Na^+ uptake rate, which was far less than net Cl^- uptake rate in the gut of the traira (Fig. 6). This charge discrepancy was too great to be explained by the accompanying K^+ and NH_4^+ (approximately equivalent to total ammonia) flux rates (Figs. 6, 8), and deserves future investigation. A comparable discrepancy has also been seen in the freshwater killifish, *Fundulus heteroclitus* (Bucking et al. 2013). One possible explanation is the occurrence of a net secretion of HCO_3^- as seen in most seawater and some freshwater teleosts (Grosell 2011). Overall net ionic uptake rates in the intestine of the jeju are high relative to previous studies on gut sac preparations of teleosts living in more ion-rich environments (Grosell et al. 2005; Scott et al. 2006).

Gut characteristics of jeju and traira may also be directly compared with the three Amazonian serrasalmids studied by Pelster et al. (2015), because acclimation conditions were identical, and measurements were made under the same in vitro conditions to those of the present study (though K^+ fluxes were not recorded). Note that serrasalmids, like the present erythrinids, are also members of the Order Characiformes. Both the jeju and the traira are carnivorous, and this was reflected in the low gut length to fork length ratios in both species (Table 1), which were even lower than the ratio in a voracious carnivore, the black piranha (*Serrasalmus rhombeus*), which lives in the same Rio Negro waters. Nevertheless, the total gut area per unit body mass was significantly greater in the jeju than in either the black piranha or the traira by about 50 % (Table 1), and comparable to that in the omnivorous tambaqui (*Colossoma macropomum*), though far lower than in the herbivorous pacu (*Myleus lobatus*). In general, area-specific rates of Na^+, Cl^-, fluid, ammonia, and glucose absorption were similar in the jeju relative to those in the three serrasalmids, whereas the traira differed in having much lower area-specific rates of Na^+ and Cl^- absorption (Figs. 6, 8). When the lower gut area of the traira was also taken into account, total Na^+, Cl^- (and K^+) transport capacities in the traira were markedly lower, below even those in the carnivorous black piranha, whereas Na^+ and Cl^- capacities in the jeju were comparable to those in the omnivorous tambaqui (Fig. 7). Differences in glucose, and ammonia transport capacities were not as marked, but values were generally lower in the traira and comparable to those in the black piranha (Fig. 9).

Enzyme activity measurements (Fig. 1) reinforced the higher ion transport capacity in the gut of the jeju. Intestinal ion transport processes have not been studied previously in these species, but in the intestine of the rainbow

trout, pharmacological studies have provided direct evidence for the presence of apical Na^+/H^+ exchange and Na^+ channel/v-type H^+ATPase as mechanisms for Na^+ uptake across the intestinal tract (Nadella et al. 2014). The former depends on basolateral Na^+,K^+ATPase activity, and the latter on apical v-type H^+-ATPase activity, so both enzymes were assayed in the present study. Both were higher overall in the gut of the facultative air-breather, the jeju, in accordance with the higher net absorptive rates. However, we cannot eliminate the possibility that these differences are related to differences in digestive processes.

Conclusions and perspectives

Surprisingly, there appear to be no previous investigations directed at the specific question of what happens to ionoregulatory capacity at gills versus gut during the evolutionary transition to air-breathing. This contrasts with previous considerations of the ionoregulatory capacities of skin and kidney in the transition process (see "Introduction"). Overall, our results largely confirm our initial hypotheses, showing lower ion uptake capacity at the gills and higher ion uptake capacity at the intestine in the facultatively air-breathing species. Therefore we speculate that the development of this greater gut uptake capacity, as well as the availability of appropriate ion-rich foods, may have been a precondition for evolution of increased reliance on air-breathing. Interestingly, the capacity to air-breathe in the jeju seems to insulate its remaining gill ionoregulatory function from fluctuations in environmental oxygen levels. Thus it is able to avoid the "osmo-respiratory compromise" seen in many water-breathing fish whereby increases in O_2 uptake demands placed on the gills result in changes in effective branchial permeability (e.g. increased branchial perfusion and surface area, decreased diffusion distance) which are unfavourable to iono- and osmoregulation (e.g. Randall et al. 1972; Iftikar et al. 2010; Robertson et al. 2015). Our conclusion is based on a comparison of fasted individuals of only two closely related teleost fish living in extremely ion-poor water. In future, it will be of interest to extend this idea to tests in other water chemistries, other species groups, and with the provision of food. In another Amazonian teleost living in similarly ion-poor water, the oscar (*Astronotus ocellatus*), which is an obligate water-breather, the availability of ions from food resulted in decreased Na^+ uptake and a negative net Na^+ balance at the gills (De Boeck et al. 2013). Indeed, does the availability of ions from food influence air-breathing behaviour? Is air-breathing more prevalent in carnivorous species than in herbivores, due to the higher Na^+ and Cl^- content of the prey? If forced to ventilate more air and less water, do bimodal breathers increase ionoregulatory capacity at the gut? These are all interesting questions for future investigation.

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