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The consequences of reversible gill remodelling on ammonia excretion in goldfish (*Carassius auratus*)

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SUMMARY

Goldfish acclimated to cold water (e.g. 7°C) experience a marked reduction in functional lamellar surface area owing to the proliferation of an interlamellar cell mass (ILCM), a phenomenon termed gill remodelling. The goal of the present study was to assess the consequences of the reduced functional surface area on the capacity of goldfish to excrete ammonia. Despite the expected impact of ambient temperature on functional surface area, fish acclimated to 7°C and 25°C exhibited similar rates of ammonia excretion ($J_{net,amm}$); the Q_{10} values for fed and starved fish were 1.07 and 1.20, respectively. To control for possible temperature-related differences in rates of endogenous ammonia production, $J_{net,amm}$ was determined at the two acclimation temperatures after loading fish with 1.12 μmol g^{-1} of NH_4Cl. In the 3 h post-injection period, $J_{net,amm}$ was elevated to a greater extent in the 25°C fish. To estimate the potential contribution of increased ventilation and cardiac output to ammonia clearance in the warmer fish, the ammonia loading experiment was repeated on the 7°C fish immediately after they were exercised to exhaustion. The rate of excretion of ammonia was significantly increased in the exercised 7°C fish (presumably experiencing increased ventilation and cardiac output for at least some of the measurement period) suggesting that differences in external and internal convection may at least partially explain the enhanced capacity of the 25°C fish to clear the ammonia load. To more specifically assess the contribution of the different functional surface areas on the differing rates of ammonia clearance at the two acclimation temperatures, the 7°C fish were exposed for 7 days to hypoxia (P_{O_2}=10 mmHg=1.33 kPa), a treatment known to cause the disappearance of the ILCM. The results demonstrated that the hypoxia-associated loss of the ILCM was accompanied by a significant increase in the rate of ammonia clearance in the 7°C fish when returned to normoxic conditions. To determine whether compensatory changes in the ammonia transporting proteins might be contributing to sustaining $J_{net,amm}$ under conditions of reduced functional lamellar surface area, the relative expression and branchial distribution of four Rh proteins were assessed by western blotting and immunocytochemistry. Although the relative expression of the Rh proteins was unaffected by acclimation temperature, there did appear to be a change in the spatial distribution of Rhag, Rhbg and Rhcg1. Specifically, these three Rh proteins (and to a lesser extent Rhcg2) appeared to localize in cells on the outer edge of the ILCM that were enriched with Na^+/K^+-ATPase. Thus, we suggest that despite the impediment to ammonia excretion imposed by the ILCM, goldfish acclimated to 7°C are able to sustain normal rates of excretion owing to the redistribution of ammonia transporting cells.

Key words: gill, Rh proteins, interlamellar cell mass, osmorespiratory compromise, nitrogen metabolism.

INTRODUCTION

Ammonia excretion in fish occurs predominantly at the gill with only a minor fraction (<10%) being excreted by the kidney (Maetz, 1972; Wood, 1993). Traditionally, ammonia (referring to the sum of NH_3 and NH_4^+) excretion across the apical membrane of gill epithelial cells was thought to reflect variable contributions of passive diffusion of NH_3 through the lipid bilayer and electroneutral Na^+/NH_4^+ exchange (Maetz and Garcia Romeu, 1964; Maetz, 1973) (for reviews, see Walsh and Henry, 1991; Wright, 1995; Wilkie, 2002). However, in recent years, an alternative model has emerged in which the transepithelial diffusion of NH_3 and/or NH_4^+ is facilitated by the presence of ammonia channels formed by Rh (Rhesus) proteins (Hung et al., 2007; Nawata et al., 2007; Nakada et al., 2007a; Nakada et al., 2007b; Shih et al., 2008; Hung et al., 2008; Tsui et al., 2009; Braun et al., 2009b) (see reviews by Weihrauch et al., 2009; Wright and Wood, 2009). Experiments on zebrafish (*Danio rerio*) using gene knockdown techniques (Braun et al., 2009a) have demonstrated that a significant fraction of ammonia excretion (~50%) in larvae is probably occurring *via* the Rh proteins Rhag, Rhbg and Rhcg1. In pufferfish (*Takifugu rubripes*) (Nakada et al., 2007b) and adult zebrafish (Nakada et al., 2007a; Braun et al., 2009b), the branchial Rh proteins are arranged in a precise spatial sequence to allow the step-by-step movement of ammonia from the blood into the water across a discrete series of barriers. Thus, Rhcg1 and Rhcg2 are localized to the apical membranes of ionocytes and pavement cells, respectively whereas Rhbg is confined to the basolateral membranes of pavement cells. The initial step for ammonia entry into the lamellar epithelium is facilitated by Rhag which is localized to pillar cells (Nakada et al., 2007b) that form the vascular channels within the gill lamellae (Laurent and Dunel, 1980).

Regardless of the relative contributions of passive NH_3 diffusion, Na^+/NH_4^+ exchange and Rh protein-facilitated NH_3/NH_4^+ movement, the net rate of ammonia excretion across the gill should be positively correlated with functional lamellar surface area, all else being equal. While changes in functional lamellar surface area can be mediated acutely *via* cardiovascular adjustments (Booth, 1979; Farrell et al., 1980), it is also clear that several fish species can chronically modify gill functional surface area by structural

remodelling (for reviews, see Sollid and Nilsson, 2006; Nilsson, 2007). For example, crucian carp (*Carassius carassius*) (Sollid et al., 2003) and goldfish (*Carassius auratus*) (Sollid et al., 2005; Mitrovic et al., 2009; Mitrovic and Perry, 2009) experience a decrease in functional surface area as water temperature is lowered, which is reversible upon exposure of fish to acute hypoxia. In mangrove killifish (*Kryptolebias marmoratus*), functional surface area of the gill is reduced during aerial exposure (Ong et al., 2007). Such long-term adjustments of gill functional lamellar surface area reflects the presence or absence of a mass of cells in the interlamellar regions termed the interlamellar cell mass (ILCM) (Sollid et al., 2003). In goldfish and crucian carp, a lowering of water temperature triggers the formation of the ILCM which, in some cases, can lead to a near total covering of the gill lamellae (e.g. Sollid et al., 2003). The physiological benefit of this type of gill remodelling is probably derived from the declining energetic costs of osmoregulation because the functional loss of lamellar surface area will limit the passive loss of salts thereby reducing the need for active ionic uptake.

The physiological consequences of gill remodelling on the branchial transepithelial transfer of ions or gases are not well understood. However, from the few studies on goldfish, it would appear that fish may retain their ionoregulatory capabilities regardless of whether the lamellae are uncovered or covered because the ion transporting ionocytes migrate with the outer edge of the ILCM to maintain contact with the external environment (Mitrovic et al., 2009; Mitrovic and Perry, 2009). The present study was conceived to assess the possible consequences of gill remodelling in goldfish on the fundamental process of ammonia excretion. The toxicity of ammonia requires its effective removal yet the loss of surface area with remodelling would be expected to impede its excretion at the gill. Thus, it was hypothesized that the capacity for ammonia excretion is maintained in fish experiencing a loss of lamellar surface area owing to an increase in the expression of Rh proteins or their spatial redistribution.

MATERIALS AND METHODS

Experimental animals

All experiments were performed according to the University of Ottawa institutional guidelines which comply with those of the Canadian Council on Animal Care (CCAC). Goldfish, *Carassius auratus* Linnaeus 1758 (weighing 25.0±0.9 g, N=142) were obtained from Along's International (Mississauga, ON, Canada). They were first acclimated to 18°C in large fibreglass tanks filled with dechloraminated tap water. After at least 1 week, temperatures were either increased or decreased 1°C per day to achieve final acclimation temperatures of 7°C or 25°C. Animals were kept in the facility under a 12 h:12 h light:dark photoperiod and were fed at least once a day with commercial food pellets. Fish were kept under such conditions for at least 2 weeks prior to their use in experiments. For experiments requiring that food ration be controlled or withheld, fish were transferred to glass aquaria (20 litres) provided with water of either 7 or 25°C. To allow the long-term (7 day) acclimation to hypoxia, fish were transferred to, and held in smaller aquaria (7.5 litres). Twenty-four hours prior to any experiment, fish were moved from their holding tanks to individual boxes (approximately 600 ml water volume) provided with flowing aerated water at the appropriate acclimation temperature; fish were not fed.

Experimental protocols

Series 1 – the effects of nutritional status and temperature on ammonia excretion

Fish, acclimated to 7°C (N=16) or 25°C (N=18), were transferred to glass aquaria (20 litre) where they were fed a daily ration of 5% of their body mass for at least 6 days. This level of feeding was chosen because it was the maximal amount routinely consumed by the 7°C fish. Within each temperature group, six of the fish were fasted for 3 days. All fish were then transferred to individual boxes for a further period of 24 h when they were not fed. Thus for the fasted fish, food was withheld for a total of 4 days while the fed fish (like all experimental fish) were deprived of food for the 24-h period immediately preceding experimentation.

To monitor ammonia ($NH_3 + NH_4^+$) excretion ($J_{net,amm}$), water flow to the boxes was stopped; water temperature was maintained by immersion of the boxes in a temperature controlled bath while water oxygenation (and mixing) was provided by continuous aeration. Water samples (2 ml) were removed after a 10 min mixing period and 60 min later, frozen (–20°C) and subsequently analyzed for ammonia levels. After a 10 min period of flushing the boxes with fresh water, the entire procedure was repeated twice. The final $J_{net,amm}$ data represent the averages of the three consecutive 1-h trials. To correct for possible ammonia addition to the water from non-piscine sources, a seventh box without a fish was run as a blank. Because ammonia concentrations remained constant in the empty chambers, there was never any need to correct for exogenous ammonia production.

After the third flux period, fish were weighed after they were killed by anaesthetic overdose using a solution of benzocaine (ethyl-*P*-amino-benzoate; Sigma, St Louis, MO, USA).

Series 2 – the effects of ammonia loading

Acclimated fish (7°C, N=18; 25°C, N=18) were weighed and transferred to individual boxes 24 h prior to further experimentation. Measurements of $J_{net,amm}$ were conducted over two 1-h consecutive intervals as outlined above. Data from these two periods were averaged to provide a measure of resting $J_{net,amm}$ rates. Fish were then removed, lightly anaesthetized using benzocaine (2.4×10^{-4} mol l^{-1}) and injected intraperitoneally with 1.12 µmol g^{-1} of 140 mmol l^{-1} NH_4Cl (N=12 at each temperature); control fish (N=6 at each temperature) were injected with 1.12 µmol g^{-1} of 140 mmol l^{-1} NaCl. Fish were returned to their individual boxes and allowed to regain equilibrium and re-establish spontaneous swimming movements prior to beginning (<30 min) subsequent hourly measurements of $J_{net,amm}$ over the next 3 h. Preliminary experiments in which fish were monitored for 24 h after ammonia loading demonstrated that increases in $J_{net,amm}$ occurred only within the first 3 h. Water temperature and oxygenation were maintained as above. A separate group of fish (25°C; N=6) was injected with NH_4HCO_3 with $J_{net,amm}$ being monitored for 3 h after injection.

Series 3 – the effects of exhaustive exercise (chasing) on ammonia excretion following ammonia loading

Fish acclimated to 7°C (N=24) were weighed and transferred to individual boxes 24 h prior to further experimentation. After assessing $J_{net,amm}$ over two consecutive 1-h flux intervals (data were averaged), individual fish (N=12) were removed, placed in a 7.5 litre aquarium and then chased with a small net for 5 min until exhausted. Exhausted fish were injected with NH_4Cl, returned to their boxes and $J_{net,amm}$ was monitored hourly for a 3-h period. A group of control fish (N=12) was subjected to the same protocol except that they were not exercised.

Series 4 – the effects of hypoxia on ammonia excretion following ammonia loading

Fish acclimated to 7°C were transferred in groups of 6 to 7.5 litre aquaria provided with flowing water (0.75 l min^{-1}) delivered by a water equilibration column. For hypoxia experiments (N=12), water

P_{O_2} was reduced gradually to 10 mmHg over 24 h by bubbling the equilibration column with N_2. The P_{O_2} was maintained at 10 mmHg for a further 6 days during which time fish were fed a daily ration of 0.5% body mass (the hypoxic fish were unable to consume as much food as in Series 1). After 7 days, fish were removed, weighed and transferred to individual chambers provided with hypoxic water where they were left unfed for 24 h prior to experimentation. After measuring $J_{net,amm}$ over two consecutive 1-h intervals, fish were removed and injected with NH_4Cl after which $J_{net,amm}$ was assessed hourly over 3 h. Hypoxia was sustained during the measurements of $J_{net,amm}$ by bubbling 1.5% O_2:98.5% N_2 through the water in the fish boxes using a two-channel gas mixer (Cameron Instruments Inc., Port Aransas, TX, USA). A separate group of control fish (N=12) was subjected to the same protocol except that they were maintained continually under normoxic conditions. A final group (N=12) was subjected to hypoxia for 7 days but then allowed to recover under normoxic conditions for 24 h prior to ammonia loading.

Series 5 – the effect of temperature on Rh protein expression levels and localization within the gill

Fish acclimated to 7°C or 25°C (N=6 for each group) were removed from holding tanks, killed by a blow to the head and the gills dissected for analysis of Rh protein levels by western blotting or protein localization within the tissues by immunocytochemistry.

Western blotting

Total protein was prepared by homogenizing dissected goldfish gills on ice and subsequently incubating homogenate in protein extraction buffer [10 mmol l^{-1} Tris (pH 7.4), 2% Triton X-100 with complete protease inhibitor cocktail (Roche, Mississauga, ON, Canada)]. Samples were centrifuged at 14,000 ***g*** for 20 min at 4°C and supernatants were stored at –20°C. The concentration of protein in each sample was determined using a bicinchoninic acid protein assay with BSA as the standard. After adding 2× Laemmli loading buffer (Bio-Rad, Mississauga, ON, Canada) and 2-mercaptoethanol (5% v/v), samples (75 µg per lane) were size-fractioned using 10% SDS-PAGE gel at 200 V. Following electrophoresis, proteins were transferred onto PVDF membranes (Bio-Rad) at 15 V for 1 h using a semi-dry transblot unit (Bio-Rad). After transfer, membranes were blocked in 5% milk–TBST (1× Tris-buffered saline with 0.05% Tween 20) for 3 h at room temperature, and then incubated with one of four primary antibodies diluted in 1% milk–TBST (1:2000) at room temperature overnight: rabbit polyclonal antibodies to the Rh proteins, Rhag, Rhbg and Rhcg2, were raised in *Fugu* (Nakada et al., 2007b) and a rabbit polyclonal antibody to Rhcg2 was raised against the zebrafish protein (Nakada et al., 2007a). The *Fugu* Rh protein antibodies were previously shown to cross react specifically with zebrafish proteins (Braun et al., 2009a). Following three 5-min washes in TBST, membranes were incubated with HRP-conjugated anti-rabbit IgG secondary antibody (Bio-Rad) diluted in 1% milk–TBST (1:5000) for 1 h at room temperature. After incubation, membranes were washed four times for 10 min each in TBST. Bands were visualized using chemiluminescence substrates (Pico or Femto; Pierce, Rockford, IL, USA). Images were acquired by ChemiDoc equipped with Quantity-One 1-D analyzer software (Bio-Rad).

After probing with one of the antibodies against Rh proteins, membranes were stripped using Re-Blot Plus (Chemicon International Inc., Millipore, Billerica, MA, USA) and re-probed with a mouse monoclonal antibody raised against *Tetrahymena thermophilia* α-tubulin (12G10; Developmental Studies Hybridoma Bank, University of Iowa) with the following modification. Membranes were blocked with 5% milk–TBST for 1 h, incubated with primary antibody (1:1000) for 2 h and incubated with HRP-conjugated anti-mouse IgG antibody (1:5000) for 1 h. The density of protein bands was determined using ImageJ software (NIH). To control for differences in protein loading, the densities of the Rh protein bands were normalized to α-tubulin band densities.

Immunocytochemistry

Dissected gill arches were fixed overnight at 4°C in 4% paraformaldehyde (PFA) prepared in PBS. The samples were then cryoprotected by sequentially incubating in 15% and 30% sucrose solutions (in PBS). The gills were embedded in OCT cryo-sectioning medium (VWR, Mississauga, ON, Canada), incubated for 1 h at room temperature and sectioned at 10 µm thickness using a cryostat (Leica CM 1850 Laboratories Eq., Nussloch, Germany). Sections were collected on SuperFrost Plus glass slides (Fisher Scientific, Ottawa, ON, Canada).

Sections were baked at 42°C for 15 min for simplified heat-induced epitope retrieval. Sections were then rehydrated in PBS for 5 min, and blocked with 5% goat serum in PBST (0.5% Triton X-100 in PBS) for 1 h at room temperature. To remove residual Triton X-100, slides were washed three times for 5 min with PBS and incubated with one of the following primary antibodies (α5, Rhag, Rhbg, Rhcg1 or Rhcg2) overnight at room temperature. α5, a mouse monoclonal antibody raised against the α-1 subunit of chicken Na^+/K^+-ATPase, was obtained from Developmental Studies Hybridoma Bank (University of Iowa); all antibodies were diluted at 1:100 in PBS. As a negative control for background staining, some slides were incubated with PBS lacking primary antibody. All slides were washed three times for 5 min following the incubation with primary antibody, and then incubated for 1 h in the dark at room temperature with Alexa-Fluor-conjugated secondary antibodies (1:100 in PBS) for immunofluorescence detection (Alexa-Fluor-546-conjugated goat anti-mouse IgG and Alexa-Fluor-488-conjugated anti-rabbit IgG; Molecular Probes, Burlington, ON, Canada). After three 5-min washes with PBS, DAPI-containing mounting medium (Vector Labs, Burlingame, MA, USA) was applied to the slides, to stain the nuclei, and coverslips were placed on top. Slides were observed using an Axiophot (Zeiss, Munich, Germany) microscope with an Olympus DP70 digital microscope camera and Image Pro Plus software, Version 6.0 (Media Cybernetics, Bethesda, MD, USA).

Analytical procedures

Water samples were analyzed for ammonia levels using the colorimetric assay of Verdouw (Verdouw et al., 1978) modified for microplate use. Absorbance at 650 nm was determined using a plate reader (Devices Spectramax Plus 384, Concord, ON, Canada).

Water P_{O_2} was measured using a Clark type O_2 electrode connected to an O_2 meter (Cameron Instruments, Port Aransas, TX, USA). The analogue P_{O_2} data were acquired continually using Biopac (Harvard Instruments, Montreal, QC, Canada) data acquisition hardware and software (AcqKnowledge, version 3.7.3). The O_2 electrode was calibrated using a zero solution (2% sodium sulphite) and air-equilibrated water maintained at the appropriate acclimation temperature.

ILCM analysis

The first right gill arch of each fish was removed and fixed in 4% paraformaldehyde (PFA) until it was ready for sectioning. The gills were placed in 30% sucrose (w/v) solution 24 h before sectioning to dehydrate and cryoprotect the tissue. Prior to sectioning they were

placed in OCT Cryomatrix (Thermo Scientific, Inc., Ottawa, ON, Canada) for at least 1 h before being frozen at –30°C. The tissue sample was then sliced into 12–14 μm thick sections using a Leica CM3050 (Leica Microsystems, Inc., Richmond Hill, ON, Canada) cryostat and stained with Haematoxylin (Harris Hematoxylin with glacial acetic acid; Poly Scientific, Bay Shore, NY, USA) to visualize the tissue. Using light microscopy, photos of six gill filaments were taken randomly per section for six sections per fish (36 photos per fish). An Axiophot (Zeiss, Germany) microscope with an Olympus DP70 digital camera were used to obtain the images and the relative cross sectional area of the ILCM was determined using Image Pro version 6.0 and Image-J software (http://rsbweb.nih.gov/ij/index.html).

Statistical analyses

All data are presented as means ± 1 s.e.m. Ammonia excretion data were analyzed either by two-way ANOVA (Series 1), repeated measures two-way ANOVA (Series 2–4) or unpaired Student's *t*-test (Series 5). If appropriate, *post hoc* multiple comparison tests were performed after ANOVA to identify the significantly different data points (Bonferroni *t*-test). All statistical analyses were performed using a commercial software package (SigmaStat version 3.5; SPSS Inc.).

RESULTS

Series 1 – the effects of nutritional status and temperature on ammonia excretion

At both acclimation temperatures, there was a pronounced effect of feeding history on $J_{net,amm}$ with the fed fish exhibiting markedly higher excretion rates ($P<0.001$; Fig. 1). Regardless of nutritional status, however, acclimation temperature did not significantly influence the rates of $J_{net,amm}$ ($P=0.091$; Fig. 1); over the 18°C temperature range, the Q_{10} values for fed and starved fish were only 1.07 and 1.20, respectively.

Series 2 – the effects of ammonia loading

Ammonia excretion was unaffected by handling and physical manipulation because intraperitoneal injections of NaCl at both acclimation temperatures (Fig. 2A) was without effect ($P=0.70$).

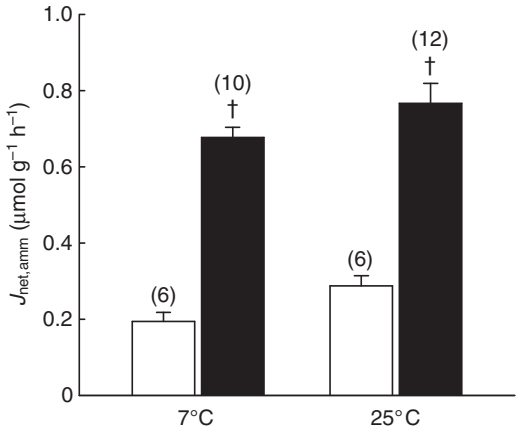

Fig. 1. The effects of acclimation temperature (7 or 25°C) and feeding history on whole body ammonia excretion ($J_{net,amm}$) in goldfish (*Carassius auratus*). Fish were either fasted (unfilled bars) or fed the equivalent of 5% body mass per day (filled bars). Data are shown as means ± 1 s.e.m.; Numbers of fish are indicated in parentheses. †Significant differences between the fed and fasted fish ($P<0.05$).

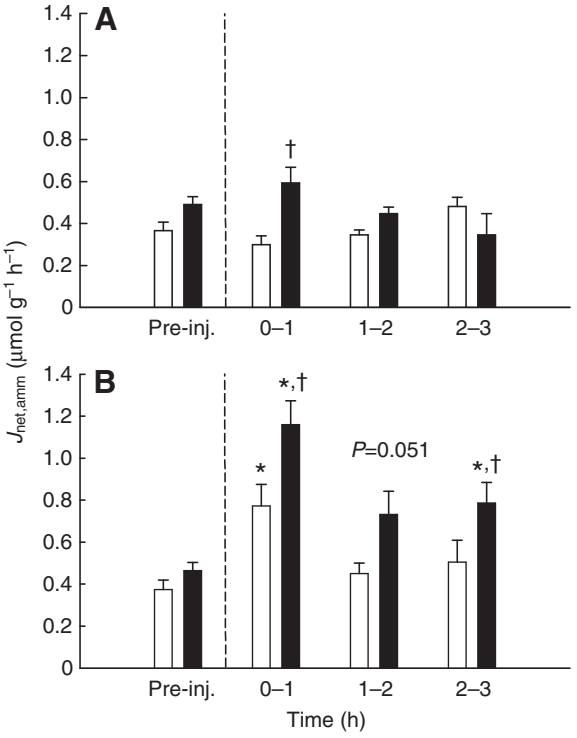

Fig. 2. The effects of intraperitoneal injections of (A) NaCl or (B) NH_4Cl (1.12 μmol g^{-1} of 140 mmol l^{-1} NaCl or NH_4Cl) on whole body ammonia excretion ($J_{net,amm}$) in goldfish (*Carassius auratus*) acclimated to 7°C (unfilled bars; $N=18$) or 25°C (filled bars; $N=18$). Data are shown as means ± 1 s.e.m.; *statistically different ($P<0.05$) from the pre-injection (Pre-inj.) values; †statistical difference ($P<0.05$) between the 7 and 25°C acclimated fish.

Thus, the significant increases ($P<0.001$) in $J_{net,amm}$ following the injection of NH_4Cl (Fig. 2B) were a specific consequence of internal ammonia loading. The clearance of the ammonia load was markedly enhanced ($P<0.001$) in the fish acclimated to 25°C (Fig. 2B). At 25°C, the total amount of ammonia excreted over 3 h was 1.00±0.20 μmol g^{-1} or roughly double the amount cleared at 7°C (0.45±0.08 μmol g^{-1}).

Injecting fish with equimolar quantities of NH_4HCO_3 (data not shown) caused $J_{net,amm}$ to increase in an identical manner to that of NH_4Cl injection (e.g. $J_{net,amm}$ was increased to 1.13±0.1 μmol g^{-1} h^{-1} during the first measurement period following NH_4HCO_3 injection in comparison with 1.16±0.1 μmol g^{-1} h^{-1} following NH_4Cl injection).

Series 3 – the effects of exhaustive exercise (chasing) on ammonia excretion following ammonia loading

Prior chasing until exhaustion of fish acclimated to 7°C resulted in greater $J_{net,amm}$ in the first 1 h of measurement following the injection of NH_4Cl (Fig. 3); $J_{net,amm}$ increased from 0.34 to 0.67 μmol g^{-1} h^{-1} in the control fish and from 0.34 to 1.02 μmol g^{-1} h^{-1} in the exercised fish.

The size of the ILCM was unaffected by the chasing protocol; the extent of ILCM coverage (as a percentage of total interlamellar area) was 83.2±5.0 ($N=6$) and 75.6±3.3% ($N=6$) in the control and exercised fish, respectively (data not shown).

Series 4 – the effects of hypoxia on ammonia excretion following ammonia loading

Fish exposed to hypoxia for 1 week exhibited a significantly greater rate of ammonia clearance after ammonia loading when assessed

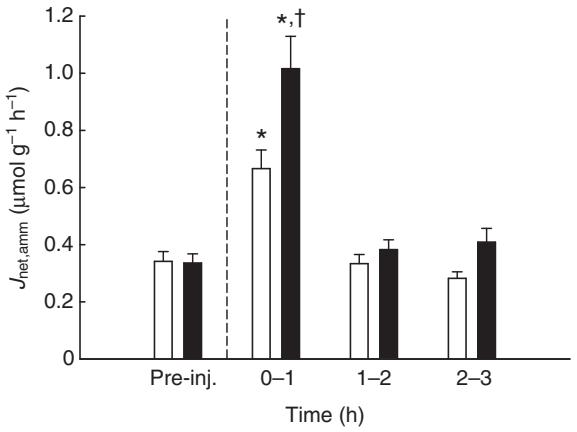

Fig. 3. The effects of intraperitoneal injections of NH_4Cl (1.12 μmol g^{-1} of 140 mmol l^{-1} NH_4Cl) on whole body ammonia excretion ($J_{net,amm}$) in goldfish (*Carassius auratus*) acclimated to 7°C under normal conditions (unfilled bars; *N*=12) or after being exercised until exhaustion (filled bars; *N*=12). Data are shown as means ± 1 s.e.m.; *statistically different (*P*<0.05) from the pre-injection (Pre-inj.) values; †statistical differences (*P*<0.05) between the control and exercised fish.

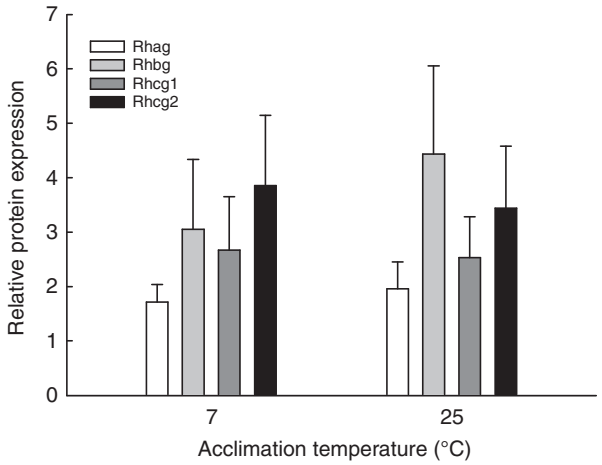

Fig. 5. The relative protein expression (normalized to α-tubulin) of Rh glycoproteins in the gills of goldfish (*Carassius auratus*) acclimated to 7 or 25°C (*N*=6 at each temperature). Data are shown as means ± 1 s.e.m.

under continuing conditions of hypoxia (Fig. 4). To eliminate the potential contribution of hypoxia itself to ammonia clearance, the experiment was repeated on fish that were returned to normoxia for 24 h. In these fish, the rate of ammonia clearance was also higher than in control fish (~twofold higher; Fig. 4) and was similar to the rate of ammonia clearance observed in the fish acclimated to 25°C. There was no difference in the rate of ammonia clearance between the previously hypoxic fish assessed under normoxic or hypoxic conditions (*P*=0.084; Fig. 4).

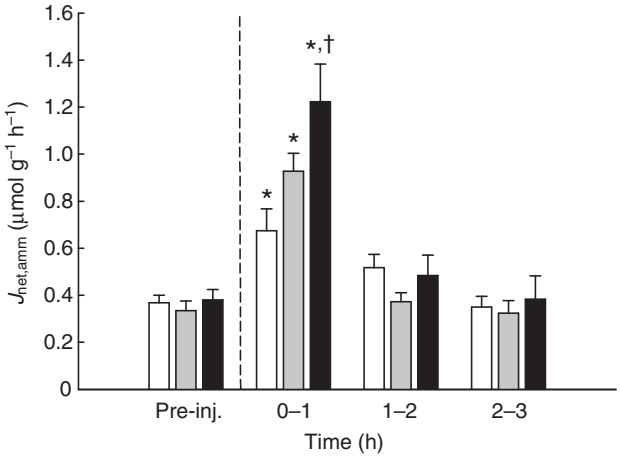

Fig. 4. The effects of intraperitoneal injections of NH_4Cl (1.12 μmol g^{-1} of 140 mmol l^{-1} NH_4Cl) on whole body ammonia excretion ($J_{net,amm}$) in goldfish (*Carassius auratus*) acclimated to 7°C under control (normoxic conditions (unfilled bars; *N*=12) or after 1 week of exposure to hypoxia (shaded bars; *N*=12). A final group of fish (solid filled bars; *N*=12) were exposed to hypoxia for 1 week but then recovered in normoxic water prior to ammonia loading. Data are shown as means ± 1 s.e.m.; *statistical differences (*P*<0.05) from the pre-injection (Pre-inj.) values; †statistical differences (*P*<0.05) from the control fish.

Series 5 – the effect of temperature on Rh protein expression levels and localization within the gill

The relative expression levels in the gill of the four Rh proteins, as assessed by western blotting, remained constant regardless of acclimation temperature (Fig. 5). However, the pattern of their distribution within the gill appeared to be modified by the gill remodelling associated with temperature change. As previously shown in pufferfish (Nakada et al., 2007b) and zebrafish (Braun et al., 2009b), Rhag was localized to the pillar cell membranes (Fig. 6) presumably to act as the initial exit pathway for ammonia from the lamellar blood channels to the lamellar epithelial cells. Additionally, some lamellar epithelial cells exhibited Rhag immunoreactivity; these cells did not appear to be enriched with Na^+/K^+-ATPase. A subset of the Na^+/K^+-ATPase-enriched cells on the filament epithelium exhibited Rhag immunoreactivity (Fig. 6C). With the profound remodelling of the gill in fish acclimated to 7°C, the Na^+/K^+-ATPase-enriched cells were redistributed to the outer edge of the ILCM with most of them showing Rhag immunoreactivity (Fig. 6D).

In fish acclimated to 25°C, Rhbg was found at the basolateral membranes of lamellar epithelial cells, some of which were enriched with Na^+/K^+-ATPase (Fig. 7B). With the appearance of the ILCM at 7°C, the lamellar localization of Rhbg was maintained but there also appeared to be distinct localization to the Na^+/K^+-ATPase-enriched cells of the outer edge of the ILCM as well as a more diffuse distribution within the cell mass itself (Fig. 7C).

Rhcg1 was distributed along the lamellar epithelium where it appeared to be specifically localized to the apical membranes of all cells regardless of whether or not they were enriched with Na^+/K^+-ATPase (Fig. 8B,C). With the redistribution of the Na^+/K^+-ATPase-enriched cells to the outer edge of the ILCM at 7°C, the pattern of lamellar staining was retained, yet there was less evidence of co-localization of Rhcg1 and Na^+/K^+-ATPase (e.g. Fig. 8D). Occasionally, however, higher magnification images did reveal some Rhcg1 immunoreactivity in the Na^+/K^+-ATPase-enriched cells of the ILCM outer edge (Fig. 8E). The distribution pattern of Rhcg2 within the gill resembled that of Rhcg1 (Fig. 9).

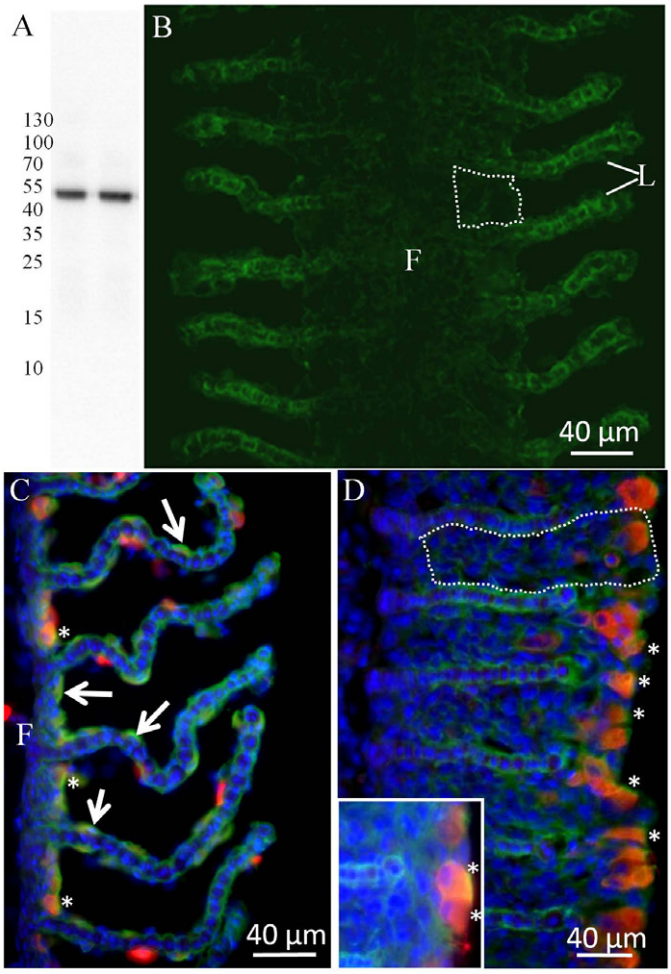

Fig. 6. (A) A representative western blot and (B–D) representative immunofluorescence micrographs illustrating the branchial distribution of Rhag (green fluorescence) and Na^+/K^+-ATPase (NKA; red fluorescence; C and D only) in goldfish (*Carassius auratus*) acclimated to 25°C (B,C) or 7°C (D). Nuclei were stained with DAPI (blue fluorescence). The western blot revealed a single immunoreactive bands at approximately 50 kDa in two lanes loaded with gill protein from different fish. The dotted lines in B and D indicate representative interlamellar cell masses (ILCM) at the two acclimation temperatures. In addition to the obvious localization of Rhag to pillar cells, staining was also observed on lamellar epithelial cells either singly (arrows) or colocalized with NKA on cells on the filament (F) or outer edge of the ILCM (asterisks); L, lamella. The inset in D shows a high magnification image of a cell on the outer edge of the ILCM co-expressing NKA and Rhag.

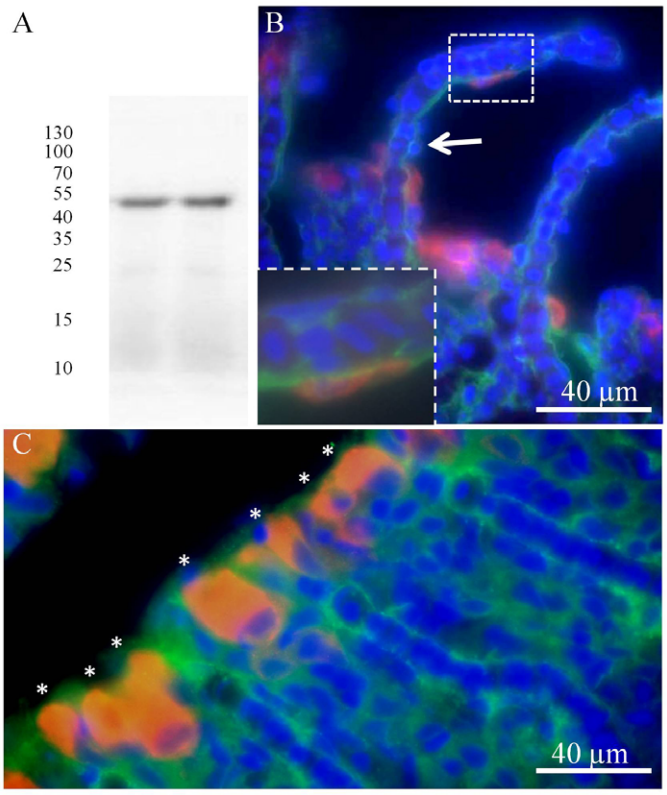

Fig. 7. (A) A representative western blot and (B,C) representative immunofluorescence micrographs illustrating the branchial distribution of Rhbg (green fluorescence) and Na^+/K^+-ATPase (NKA; red fluorescence) in goldfish (*Carassius auratus*) acclimated to 25°C (B) or 7°C (C). The nuclei were stained with DAPI (blue fluorescence). The western blot revealed single immunoreactive bands at approximately 50 kDa in two lanes loaded with gill protein from different fish. (B) In fish acclimated to 25°C, Rhbg was present at the basolateral membranes of lamellar epithelial cells (arrow), some of which were enriched with NKA (inset in B). (C) With the appearance of the ILCM at 7°C, the lamellar localization of Rhbg was maintained but there also appeared to be distinct localization to the NKA-enriched cells (asterisks) of the outer edge of the ILCM as well as a more diffuse distribution within the cell mass itself (Fig. 7C).

DISCUSSION

The results of the present study demonstrate that, despite the profound structural remodelling of the gill that occurs during acclimation to cold water (7°C), goldfish are able to sustain ammonia excretion. Thus, although the results have demonstrated that the ILCM presents a barrier for ammonia excretion, one or more compensatory factors are apparently contributing to permit normal rates of excretion. The present findings demonstrate that the capacity of goldfish to excrete ammonia in the face of a diminished lamellar surface area may reflect, at least in part, a spatial redistribution of the branchial ammonia-transporting Rh proteins. Clearly, the continued ability of goldfish to excrete ammonia in the face of the impediment posed by the ILCM is crucial to the success of thermally induced gill remodelling as a strategy to conserve energy.

In addition to modifying surface area, acclimation of goldfish to differing temperatures is expected to influence a myriad of other factors that could potentially influence the rates of branchial ammonia excretion. Such temperature-dependent processes include metabolic rate, food consumption, nitrogen metabolism, ventilation and cardiac output. Thus, a challenge in the present study was to distinguish between the specific effects of reduced surface area and these other temperature-dependent changes on rates of ammonia excretion during thermally induced gill remodelling. This was achieved by comparing fed and fasted fish and by assessing ammonia clearance rates after loading with equimolar quantities of NH_4Cl. In addition, ammonia excretion was assessed in fish maintained at a constant temperature (7°C) while manipulating convection (following exhaustive exercise) or functional lamellar surface area (following exposure to hypoxia). Although increased perfusion of more distal lamellae during intense exercise might also be expected to increase functional surface area, the presence of an ILCM in the fish acclimated to cold water would presumably lessen the impact of such a response because the distal lamellae are covered. The results of these experiments revealed that the ability to excrete

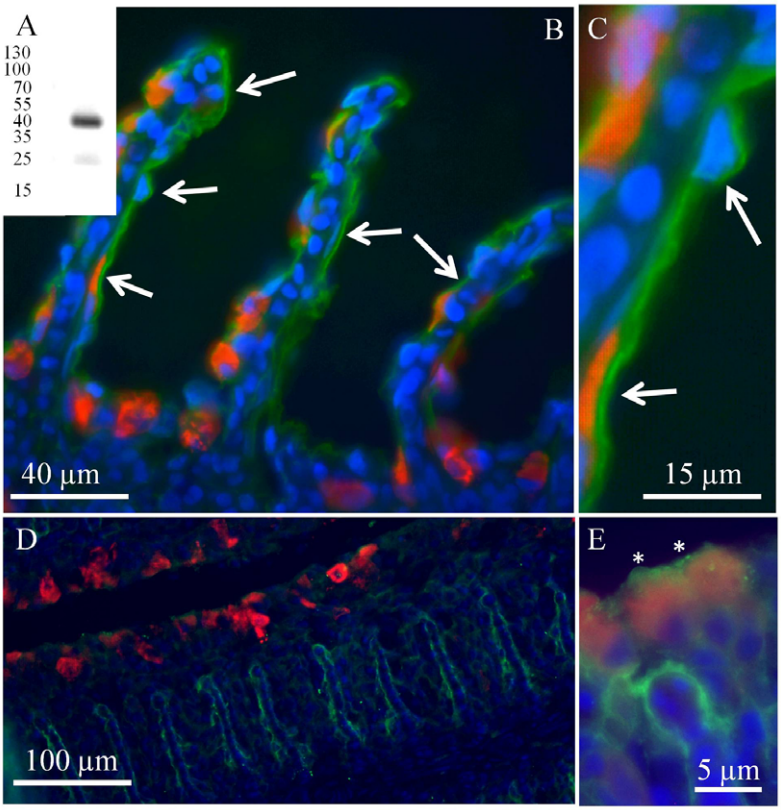

Fig. 8. (A) A representative western blot and (B–E) representative immunofluorescence micrographs illustrating the branchial distribution of Rhcg1 (green fluorescence) and Na^+/K^+-ATPase (NKA; red fluorescence) in goldfish (*Carassius auratus*) acclimated to 25°C (B,C) or 7°C (D,E). The nuclei were stained with DAPI (blue fluorescence). The western blot revealed a single immunoreactive band at approximately 40 kDa. In fish acclimated to 25°C (C, Rhcg1 was present at the apical membranes of lamellar epithelial cells (arrows), some of which were enriched with NKA. With the redistribution of the NKA-enriched cells to the outer edge of the ILCM at 7°C, the pattern of lamellar staining was retained yet there was less evidence of colocalization of Rhcg1 and NKA (D). Occasionally, however, higher magnification images did reveal some Rhcg1 immunoreactivity in the NKA-enriched cells of the outer edge of the ILCM (E, asterisks).

the exogenous ammonia load in the cold-acclimated fish was probably being constrained both by reduced rates of gill ventilation and perfusion as well as by a decrease in functional lamellar functional surface area.

The effects of acclimation temperature on ammonia excretion

The results of previous studies have demonstrated that the effects of temperature on ammonia excretion are complex and subject to the confounding influences of temperature on feeding, metabolic rate and the activities of several key catabolic proteins (Wood, 2001; Ballantyne, 2001). However, in studies on rainbow trout that have simultaneously measured feeding, nitrogen excretion and protein synthesis, it is clear that ammonia excretion decreases with falling temperature owing to the combined effects of decreased food consumption, protein synthesis and overall metabolic rate (Wood, 2001). In the present study, the effects of temperature acclimation on ammonia excretion were assessed in fed and fasted fish. The fed normoxic fish were given a common food ration of 5% body mass per day which corresponded to near maximal rates of food consumption for the 7°C fish but probably meant that the 25°C fish were being subjected to dietary restriction relative to the fish in cold water. Thus, as a proportion of overall metabolic rate, it is likely that the fish acclimated to 25°C were receiving fewer calories. We considered this to be less problematic than comparing fish with differing absolute caloric intakes. Under this condition of matched dietary food intake there was no significant effect of the 18°C temperature change on rates of ammonia excretion; the Q_{10} value was 1.07. This result is in marked contrast to a Q_{10} value of 4.0 reported for goldfish acutely transferred from 16 to 6°C (Maetz, 1972); fish were not fed for at least 24 h in those experiments. Because fish in that same study injected with NH_4Cl exhibited a lower average Q_{10} of 1.9 (compared with 4.0), it was concluded that a significant component of the reduced ammonia excretion with falling temperature was caused by a reduced rate of nitrogen metabolism (Maetz, 1972). The fasted fish in the present study exhibited a Q_{10} of 1.20 which is low relative to other studies (Wood, 2001) and surprising given that these fish were also experiencing a reduction in functional lamellar surface area, a possible decrease in the rate of endogenous ammonia production and reduced rates of ventilation and perfusion. Although the results of these experiments would appear to indicate that the formation of an ILCM in the cold-acclimated fish does not impede ammonia excretion, this conclusion should be treated with caution (see below) owing to the confounding effects of temperature on metabolism and physiological function. Thus, additional experiments were performed to control for ammonia production and to assess the potential roles of hyperventilation and perfusion on rates of ammonia excretion.

The effects of acclimation temperature on ammonia excretion after ammonia loading

To control for altered rates of ammonia production, ammonia excretion was monitored in fish that were injected intraperitoneally with NH_4Cl. Because loading with NH_4Cl is likely to cause metabolic acidosis (Cameron and Kormanik, 1982), subsequent increases in ammonia excretion could reflect the need to excrete the exogenous ammonia load (*via* any route) as well as a specific increase in Na^+/NH_4^+ exchange triggered by the metabolic acidosis. To ensure that fish were responding to the ammonia load rather than metabolic acidosis, a separate series of experiments was performed in which fish were injected with an equimolar quantity of NH_4HCO_3, a protocol expected to cause metabolic alkalosis. Because the increases in ammonia excretion following loading were identical regardless of whether NH_4Cl or NH_4HCO_3 was used, it is

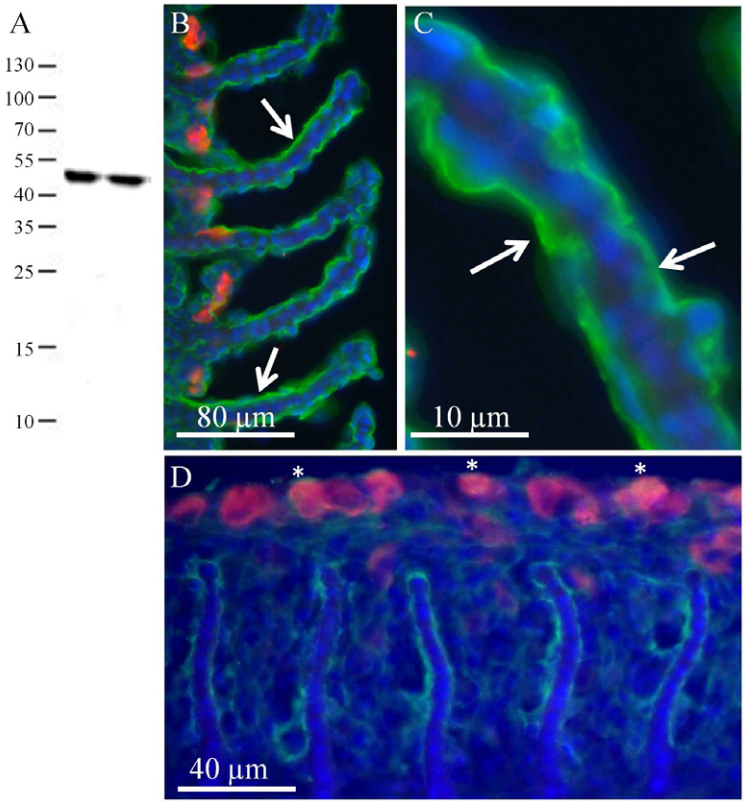

Fig. 9. (A) A representative western blot and (B–D) representative immunofluorescence micrographs illustrating the branchial distribution of Rhcg2 (green fluorescence) and Na^+/K^+-ATPase (NKA; red fluorescence) in goldfish (*Carassius auratus*) acclimated to 25°C (B,C) or 7°C (D). The nuclei were stained with DAPI (blue fluorescence). The western blot revealed single immunoreactive bands at approximately 50 kDa in two lanes loaded with gill protein from different fish. In fish acclimated to 25°C (B,C), Rhcg1 was present at the apical membranes of lamellar epithelial cells (arrows), some of which were enriched with NKA. With the redistribution of the NKA-enriched cells to the outer edge of the ILCM at 7°C, the pattern of lamellar staining was retained yet there was less evidence of colocalization of Rhcg2 and NKA. Occasionally, however, higher magnification images did reveal some Rhcg2 immunoreactivity in the NKA-enriched cells of the outer edge of the ILCM (D, asterisks).

clear that fish were responding predominantly to the increasing levels of ammonia rather than a changing blood acid–base status.

The results clearly demonstrated that the fish acclimated to cold water were unable to clear the exogenous ammonia load as effectively as the fish acclimated to warm water (Fig. 2B). The warm water fish were able to excrete 90% of the injected ammonia load within the 3 h measurement period compared with only 40% in the fish acclimated to cold water. Although these data are consistent with the ILCM acting as a barrier to ammonia excretion, it is also possible that the slower clearance of ammonia in the cold-acclimated fish reflects reduced ventilation volume and/or cardiac output.

The effects of exhaustive exercise on ammonia clearance in fish acclimated to 7°C

In an attempt to ascertain whether the decreased levels of ventilation or cardiac output in the fish acclimated to cold water was limiting ammonia excretion, fish acclimated to 7°C were exercised to exhaustion immediately prior to ammonia loading. The underlying assumption was that ventilation and cardiac output would be elevated for at least a portion of the 3 h post-injection measurement period. Although exhaustive exercise also is known to markedly increase the production of ammonia in white muscle, this additional pool contributes little to branchial ammonia excretion (at least in rainbow trout over a 4 h post-exercise interval) (Wood, 1988) because it is largely contained within the intracellular compartment (Wood, 2001). Thus, we believe that the increased clearance of ammonia in the fish subjected to exhaustive exercise reflects increasing ventilation and cardiac output in addition to other factors that might be expected to enhance excretion (e.g. increasing blood pressure, increasing circulating catecholamines) (Farrell et al., 1979; Perry and Bernier, 1999). It is interesting that similar rates of ammonia clearance were achieved in the exercised 7°C fish as in the warm water control fish despite the presence of an ILCM in the former (note that the ILCM was unaffected by the exercise treatment). It is unlikely that non-branchial ammonia excretion was significantly contributing to ammonia clearance because Maetz (Maetz, 1972) reported that the goldfish kidney could account for no more than 8% of whole body ammonia excretion after an ammonia load.

The effects of pre-exposure to hypoxia on ammonia clearance in fish acclimated to 7°C

To more specifically assess the impact of functional lamellar surface area on ammonia excretion, goldfish acclimated to 7°C were exposed to hypoxia (10 mmHg) for 7 days, a protocol that is known to cause the ILCM to disappear (Mitrovic et al., 2009). This allowed us to compare the capacity of fish at a constant temperature, but with widely differing functional lamellar surface areas, to excrete an exogenous ammonia load. The increased rates of ammonia clearance that were found to be associated with prior hypoxia exposure provide evidence that the presence of the ILCM impedes the transfer of ammonia across the gill. This finding is consistent with the results of Mitrovic et al. (Mitrovic et al., 2009) who observed an increase in the branchial efflux of polyethylene glycol (PEG 4000) in cold-acclimated goldfish experiencing a loss of the ILCM following hypoxia exposure. However, in that study (Mitrovic et al., 2009), the augmentation in PEG flux was observed only when fish were assessed under conditions of normoxia.

Given the obvious impairment of ammonia clearance associated with the presence of an ILCM, the absence of an effect of cold acclimation on the excretion of endogenous ammonia in fed or fasted fish (see above) suggests that fish were compensating for the lack of functional lamellar surface area. In this study, we tested the

hypothesis that one such compensatory mechanism was an increase in, or redistribution of, the ammonia-transporting Rh proteins.

The effects of acclimation temperature on branchial Rh protein expression

In pufferfish (*Takifugu rubripes*) (Nakada et al., 2007b) and zebrafish (*Danio rerio*) (Braun et al., 2009b), the Rh proteins are arranged spatially within the gill to provide a stepwise pathway for the excretion of ammonia from the blood to water. The movement of ammonia from the blood channels into the lamellar epithelium is believed to be facilitated by Rhag proteins localized to pillar cells whereas the translocation across the epithelium is probably enabled by Rhbg and Rhcg proteins confined to basolateral and apical membranes, respectively. In this study, we have provided evidence for a similar distribution of Rh proteins in goldfish gill, with some additional sites being identified depending on whether the ILCM was present or absent. In the fish acclimated to 25°C (no ILCM), there was also evidence of Rhag expression within epithelial cells of the lamella and filament. With the appearance of the ILCM in fish acclimated to 7°C, the expression of Rh proteins on pillar cells and lamellar epithelial cells was retained but additionally there was a striking spatial reorganization of all four Rh proteins (but in particular Rhag and Rhbg) to the outer edge of the ILCM. As demonstrated previously (Mitrovic et al., 2009; Mitrovic and Perry, 2009), there was also a profound redistribution of Na^+/K^+-ATPase-enriched cells to the outer edge of the ILCM; a subset of these Na^+/K^+-ATPase-enriched cells also expressed Rh proteins (e.g. Figs 6–9). Just as the relocation of Na^+/K^+-ATPase-enriched cells to the outer edge of the ILCM may benefit branchial Cl^- uptake (Mitrovic and Perry, 2009), the similar redistribution of Rh proteins may be a mechanism to facilitate the final step of ammonia excretion, its entry into the water. With an ILCM present, the standard localization of Rh proteins would still be required to allow ammonia passage across the lamellar epithelium but its movement throughout the ILCM would require Rh proteins (e.g. Rhbg; Fig. 7) as would its exit into the water. It would be interesting to determine whether the Rh protein-containing cells on the outer edge of the ILCM also express acid transporting proteins such as V-type H^+-ATPase or Na^+/H^+ exchangers (NHE2 or NHE3) to facilitate ammonia excretion by acid trapping (Wright et al., 1989; Shih et al., 2008; Wright and Wood, 2009). Despite the obvious redistribution of Rh proteins in fish acclimated to 7°C, there was no increase in the total quantity of these proteins as determined by western blotting.

Concluding remarks

The physical covering of the gill lamellae by an ILCM under conditions of reduced O_2 demand can be an effective strategy to reduce the energetic costs of repaying ionic losses across the gill but only if normal physiological functions can be maintained. In the present study, ammonia excretion was maintained in fish possessing covered lamellae probably as a result of the redistribution of ammonia-transporting cells to the outer edges of the ILCM where they can remain in contact with the water. This response is similar to the migration of mitochondria-rich cells to the outer edge of the ILCM that was previously reported by Mitrovic and Perry (Mitrovic and Perry, 2009) and the redistribution of cells expressing mRNA for V-type H^+-ATPase and NHE3 (Julia Bradshaw and S.F.P., unpublished data). Presumably the redistribution of ion-transporting cells is crucial to maintaining ionic homeostasis when the lamellae are covered. Finally, O_2 sensing, thought to be mediated by neuroepithelial cells within the gill filament and scattered randomly on the lamellae is believed to be sustained in fish experiencing gill remodelling because of a redistribution of neuroepithelial cells to the distal regions of the lamellae (Tzaneva and Perry, 2010). Thus, the transfer of functions from the proximal regions of lamellae to more distal regions or the edge of the ILCM appears to be a common strategy to maintain physiological processes when the gill undergoes remodelling.

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