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Branchial and extra-branchial ammonia excretion in goldfish (*Carassius auratus*) following thermally induced gill remodeling

Amanda A. Smith, Alex M. Zimmer *, Chris M. Wood

Department of Biology, McMaster University, Hamilton, Ontario, Canada L8S 4K1

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ABSTRACT

Under cold acclimated conditions, goldfish (*Carassius auratus*) express an interlamellar cell mass (ILCM) which limits diffusive ion loss but may also impede branchial ammonia excretion (J_{amm}). In the present study, goldfish were subjected to a 2-week 5 or 25 °C acclimation in order to modulate the degree of ILCM gill coverage and determine potential effects on J_{amm}. 25 °C-fish displayed gill coverage which was significantly lower than the 5 °C-fish, though the ILCM was not completely absent in these fish. 5 °C-fish demonstrated J_{amm} values approximately 60% lower than those of 25 °C-fish. The magnitude of anterior (branchial) J_{amm} strongly correlated with gill coverage ($r^2 = 0.83$), suggesting that the ILCM may impede branchial J_{amm}. Divided chamber experiments demonstrated that relative to the 25 °C-fish, 5 °C-fish relied more upon posterior routes of excretion. In response to high external ammonia (HEA; 1.5 mM NH_4HCO_3) exposures, 25 °C-fish displayed ammonia uptake while 5 °C-fish maintained excretion against HEA, suggesting that the ILCM may act as a barrier preventing ammonia uptake. In summary, the ILCM appears to impede branchial J_{amm}, such that 5 °C-rely more on extra-branchial routes of excretion. We hypothesize that gill remodeling in these fish may be intimately tied to physiological adjustments on the whole-body scale.

1. Introduction

The fish gill performs a wide variety of homeostatic control functions including ion regulation, osmoregulation, oxygen uptake, carbon dioxide removal, nitrogen excretion, and acid–base regulation (Evans et al., 2005). Much attention has been devoted to nitrogenous waste removal, with a focus on ammonia as the major end product of protein degradation. In ammoniotelic fishes, ammonia is passed through the gill lipid bilayer and into the external environment down a partial pressure gradient via facilitated diffusion through Rhesus (Rh) glycoproteins (see Weihrauch et al., 2009; Wright and Wood, 2009 for reviews). The excretion or accumulation of urea, a less toxic nitrogenous waste product, is also a route for nitrogenous waste removal by freshwater fish whereby ammonia is converted into urea within the liver as a means to avoid the potentially neurotoxic effects of accumulated plasma ammonia (Anderson, 2001; Ip et al., 2001). This is particularly apparent in zebrafish (*Danio rerio*) exposed to high external ammonia (HEA) where urea excretion (J_{urea}) was seen to significantly increase following a marked decrease in ammonia excretion (J_{amm}) (Braun et al., 2009) and in mangrove killifish (*Rivulus marmoratus*) where tissue urea levels increased during air exposure, also likely in response to decreased J_{amm} (Frick and Wright, 2002). Moreover, a number of species which express a fully functional ornithine-urea cycle (OUC) often rely on urea excretion when ammonia excretion is inhibited (Magadi tilapia, *Alcolapia grahami*, Randall et al., 1989; gulf toadfish, *Ospanus beta*, Saha and Ratha, 1990; walking catfish, *Clarias batrachus*, Walsh et al., 1990; the Indian air-breathing teleost *Heteropneustes fossilis*, Saha et al., 2002). Under normal circumstances, however, urea excretion accounts for only 5–20% of nitrogenous waste removal in typical freshwater fish (Anderson, 2001).

In the fish gill, a high surface area and short diffusion distances are beneficial for rapid and efficient gas exchange. However, as these animals are hyper-osmotic compared to their environment, water influx via osmosis coupled to diffusive ion loss is a particular problem and must be corrected by a combination of substantial urine production, efficient renal tubule reabsorption, and active ion uptake across the gill. To avoid these energetically costly processes, some fish species have developed the ability to remodel their gill structure in an attempt to reduce diffusive ion loss across the gills. (Sollid et al., 2003, 2005; Sollid and Nilsson, 2006). Under normoxic conditions, the crucian carp (*Carassius carassius*) maintains a cell mass enveloping the gill lamellae, deemed the interlamellar cell mass (ILCM), thought to prevent diffusive ion loss to the water (Sollid et al., 2003; Sollid and Nilsson, 2006). In response to hypoxia, fish gradually lose the ILCM through a combination of a lowered rate of mitosis and heightened rate of apoptosis (Sollid and Nilsson, 2006), thereby increasing

Abbreviations: HEA, high external ammonia; ILCM, interlamellar cell mass; J_{amm}, ammonia excretion; J_{urea}, urea excretion; T_{amm}, total ammonia.

* Corresponding author at: Department of Biology, McMaster University, Hamilton, Ontario, Canada L8S 4K1. Tel.: +1 905 525 9140x23237; fax: +1 905 522 6066.
E-mail addresses: smith_amanda_a@hotmail.ca (A.A. Smith), zimmea@mcmaster.ca (A.M. Zimmer), woodcm@mcmaster.ca (C.M. Wood).

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surface area by up to 7.5-fold and effectively increasing the area available for gas exchange (Sollid et al., 2003). In addition, temperature changes have also been shown to elicit similar effects (Sollid et al., 2005; Rissanen et al., 2006). As fish are ectothermic, an increase in temperature increases metabolic rate which, in turn, creates a greater demand for O_2 consumption. At high temperatures, acclimated crucian carp (25 °C) lose the ILCM, while a 5-day acclimation at low temperature (10–15 °C) promotes the regeneration of the cell mass (Sollid et al., 2005). Similarly, the goldfish (*Carassius auratus*) also demonstrates temperature-dependent ILCM coverage (Sollid et al., 2005; Mitrovic and Perry, 2009; Perry et al., 2010). Though a reduction of gill surface area in colder temperatures, where oxygen demand is lower, is beneficial in preventing diffusive ion loss, this process may consequently result in a reduction of branchial ammonia excretion if the ILCM acts as a barrier towards ammonia movement.

Perry et al. (2010) demonstrated that goldfish acclimated to a high temperature (25 °C) were able to clear an intraperitoneally injected ammonia load (either NH_4HCO_3 or NH_4Cl) at a greater rate than cold-acclimated (7 °C) fish, suggesting that the ILCM may in fact provide a physical barrier to branchial ammonia excretion. J_{amm} under normal conditions, however, was not significantly affected by temperature acclimation (Perry et al., 2010). This result is curious as fish at a higher temperature would be expected to have an increased metabolic rate and, hence, increased J_{amm} (see Wood, 2001). One potentially important factor that was not taken into account was the possible role of shunting ammonia excretion to extra-branchial routes of excretion. It is generally well-accepted that the gills account for the majority of ammonia excretion and that posterior routes (i.e., gastrointestinal, renal, and/or cutaneous routes) account for a significantly smaller proportion under normal conditions (Smith, 1929). If the ILCM impedes the branchial movement of ammonia, fish may compensate with an increase in extra-branchial J_{amm}; this has been seen in response to certain environmental conditions in other species (see Wood, 1993). Indeed, the skin may play such a role during ammonia challenges as indicated by an increase of the mRNA expression of Rh proteins in the skin of some species under ammonia-loaded conditions (Hung et al., 2007; Nawata et al., 2007; Nawata and Wood, 2009).

The goal of this study was to first investigate the potential of a 5 °C-induced ILCM acting as a barrier to ammonia movement in goldfish. We hypothesized that 5 °C-fish, in comparison to 25 °C-fish, would display a reduction in J_{amm} under normal conditions, even though Perry et al. (2010) did not show the same in their fish which were much larger in size. We further hypothesized that these fish would utilize two physiological mechanisms to deal with this ILCM-induced reduction in branchial J_{amm}: a redistribution of J_{amm} to posterior routes of excretion and/or a shift to rely more upon urea-N production and excretion to avoid ammonia toxicity. Alternatively, we hypothesized that ILCM coverage may result in an accumulation of plasma ammonia to which goldfish may be particularly resistant as studies have demonstrated these fish tolerating total plasma ammonia levels up to 14 mmol/L (Sinha et al., 2012). Finally, we predicted that during a high environmental ammonia exposure, 25 °C-fish, with a lesser degree of ILCM coverage, would show a greater ammonia uptake than 5 °C-fish, further suggesting that the ILCM acts as a barrier towards ammonia movement.

2. Materials and methods

2.1. Animals

Goldfish (*Carassius auratus*) (mass = 4.6 ± 1.3 g) were purchased from Big Al's Pet Store, Hamilton, Ontario, Canada. Fish were held in plastic tanks containing aerated, dechlorinated Hamilton tap water (moderately hard: $[Na^+] = 0.6$ mequiv/L, $[Cl^-] = 0.8$ mequiv/L, $[Ca^{2+} = 0.8$ equiv/L, $[Mg^{2+} = 0.3$ mequiv/L, $[K^+] = 0.05$ mequiv/L; titration alkalinity 2.1 mequiv/L; pH ~8.0; hardness ~140 mg/L as CaCO3 equivalents) at 5 and 25 °C. Fish were acclimated to these two temperatures for 2 weeks prior to any experimentation. Fish were fed 1% tank body mass with Big Al's brand staple fish flake. 25 °C-fish were fed daily, and 5 °C-fish were fed once every 2 days as their nutritional demand was noticeably lower at this temperature. All fish were fasted for 48 h prior to experimentation.

2.2. Whole body ammonia/urea flux (J_{amm}/J_{urea})

Fish from each acclimation temperature were chosen randomly and placed into containers containing 350 mL aerated dechlorinated, Hamilton tapwater thermostatted to the given acclimation temperature. Both groups were placed in the containers for 2 h, and 3-mL water samples were drawn from each container every 0.5 h for ammonia and urea-N analyses. pH was measured following sampling times, and was maintained at 7.6 by the addition of 0.1 M KOH or HCl.

2.3. Divided chamber apparatus

Fish were fasted for 48 h prior to experimentation. Randomly selected fish were initially anaesthetized using 125 ppm of a 1:10 clove oil in ethanol solution. Latex dams were placed directly behind the opercula, separating the gills and head from the rest of the body. The dam was then secured into place using elastic bands over a Falcon tube filled with 50 mL dechlorinated tap water of the respective acclimation temperature. This apparatus was then placed within the containers described above. A dilute clove oil anaesthetic concentration (15 ppm) was present in the water in these experiments to mildly sedate the fish in order to prevent escape from the latex dam. The effects of this anaesthetic were assessed in a separate series of experiments (see below). Containers were placed in a water bath at the given acclimation temperature. Small magnetic stir bars were placed within the weighted Falcon tube to facilitate full mixing of interior waters. Five microliters of 0.1 μCi/μL ^{22}Na was injected into the water within the Falcon tube, and a 200 μL water sample was withdrawn from the external compartment every 0.5 h to assess the integrity of the seal created by the latex dam. A maximum of 10% isotope loss from the Falcon tube to the exterior compartment, as measured by gamma counting (Perkin Elmer Wizard 1480 300 Auto Gamma Counter), was accepted as a successful dam. Water samples (3 mL) were withdrawn from the Falcon tube and exterior chamber every 30 min and pH was maintained in both chambers in the same manner as described above.

To assess the potential effects of the 15 ppm clove oil anaesthetic on ammonia and urea-N excretion, whole-body anaesthetic controls were performed for each acclimation temperature. These controls consisted of animals that were initially anaesthetized in a 125 ppm clove oil solution and then placed into individual containers described earlier which contained a 15 ppm anaesthetic solution. The dam was not used; all other experimental procedures were the same as for the whole-body excretion testing.

2.4. High external ammonia (HEA) ammonia flux (J_{amm})

Randomly selected fish were placed within containers of 350 mL aerated 1.5 mM NH_4HCO_3 in dechlorinated tap water neutralized to pH 7.6 with 0.1 M HCl. All solutions were prepared at respective acclimation temperature, and chambers were housed within water baths of the given acclimation temperature. Both groups were placed in the containers for 2 h and 1.5-mL water samples withdrawn from each container every 0.5 h for ammonia analysis. pH was maintained using methods described above.

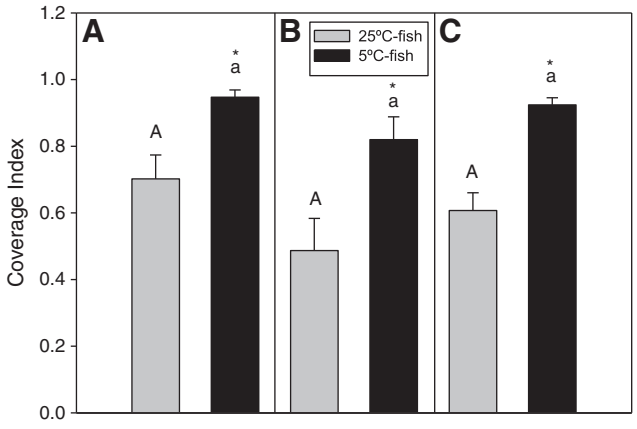

Fig. 1. Coverage indices following a 2 week acclimation period in 25 °C and 5 °C-fish (see text for scoring system). A: Whole-body control series ($n=8$). B: Whole-body anaesthetic series ($n=5$–8). C: Divided chamber series ($n=6$–8). Means sharing the same letter are not significantly different within temperature acclimation (25 °C, upper case letters; 5 °C, lower case letters). Asterisks represent significant differences between 25 °C and 5 °C-fish within each experimental series.

2.5. Blood testing

At the end of every experiment, blood samples were taken by blind caudal puncture from experimental fish using a lithium-heparinized Hamilton syringe after sacrificing the fish with a neutralized 0.1 g/L MS-222 solution. It was not possible to obtain samples from every fish. Samples were then centrifuged and the decanted plasma was stored at −80 °C.

2.6. Dissections

Following blood sampling, entire gill baskets were removed and placed on ice. Dissected gill arches were viewed under a dissecting microscope to determine the degree of coverage of the secondary lamellae by the ILCM. A total of 40 randomly selected filaments were counted on four gill arches of fish, resulting in 160 filaments counted per fish at both acclimation temperatures. The degree of coverage was scored under the following values: (0) no coverage (all lamellae of the examined filament are exposed); (1) partial coverage (the lamellae of the examined filament are only partially covered); and (2) full coverage (none of the lamellae of the examined filament are exposed). These scores were averaged out of the number of filaments counted to attain a coverage index.

2.7. Environmental scanning electron microscopy

In order to validate the light microscopy method of assessing gill coverage described above, gill coverage in a separate set of fish was viewed using environmental scanning electron microscopy (ESEM). A separate set of fish was acclimated to 25 and 5 °C using the same protocol described above and fish were treated in an identical manner throughout the acclimation period. Following 2 weeks of acclimation, three fish from each temperature acclimation were chosen randomly, sacrificed using neutralized MS-222, and two arches (one from each side) were removed and placed in Karnovsky's fixative (2% glutaraldehyde, 1% paraformaldehyde) overnight. Gill arches were then dehydrated in ascending concentrations of ethanol starting from 30% and ending with 100% before being viewed using an environmental scanning electron microscope (Philips ElectroScan 2020 ESEM, ElectroScan Corporation, Wilmington, MA, USA). Photos of 12–30 filaments per fish were captured and were scored blindly using the same scoring system described above.

2.8. Analytical techniques

Water levels of ammonia were measured using the salicylate-hypochlorite colorimetric assay (Verdouw et al., 1978). Ammonia flux rates (μmol/g/h) were calculated using the following equation:

$$J_{amm} = \left([Amm]_i - [Amm]_f\right) * (V/t * M)$$

where $[Amm]_i$ and $[Amm]_f$ are initial and final ammonia concentrations (μmol/L) between 0.5-h time (t) intervals, V is volume (L) of water within individual containers, and M is mass (g).

Plasma total ammonia concentrations (T_{amm}) were tested using an enzymatic assay kit (Raichem, Mumbai, India). Plasma clearance of ammonia (mL/g/h) was calculated using the following equation:

$$\text{Plasma clearance rate} = J_{amm}/\text{plasma } T_{amm}$$

where J_{amm} is ammonia excretion (μmol/g/h) over the 2-hour flux and plasma T_{amm} is the terminal plasma concentration of ammonia (μmol/L).

Urea-N levels were measured using a colorimetric assay (Rahmatullah and Boyd, 1980) modified for low urea concentrations.

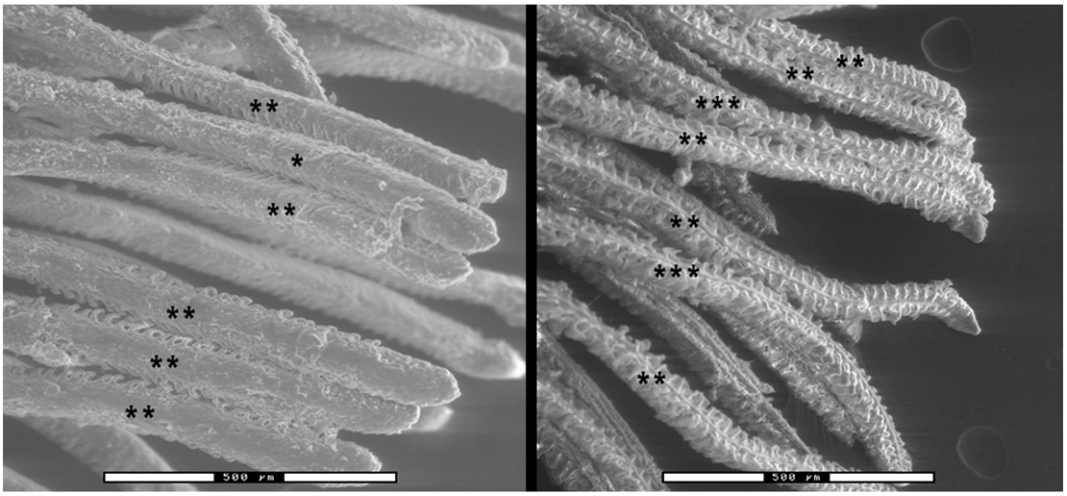

Fig. 2. Two example ESEM photos from a 25 °C-fish demonstrating fully covered filaments (single asterisks), partially covered filaments (double asterisks), and fully uncovered filaments (triple asterisks). Twenty-three photos were scored for a total of 12–30 filaments per fish.

Urea-N flux (μmol N/g/h) was determined using the following equation:

$$J_{urea} = ([Urea{-}N]_i - [Urea{-}N]_f) * (V/t * M)$$

where $[Urea\text{-}N]_I$ and $[Urea\text{-}N]_f$ are initial and final urea-N concentrations (μmol N/L) between half hour time (t) intervals, V is volume (L) of water within individual containers, and M is mass (g).

2.9. Statistics

Data are expressed as means ± 1 SEM (n = number of fish tested) and significance was accepted at $P<0.05$. Over every 2-h flux conducted in this study, a one-way ANOVA test was used (an ANOVA on ranks in the case of a failed equal variance test) to determine if there were significant differences between each 0.5-h time interval within flux measurements. In fact, no differences occurred over any flux experiment for ammonia or urea excretion and as such data reported are average excretion rates over each 2-h flux. Significance between acclimation groups within a given treatment was determined by a Student's t-test (Mann–Whitney rank sum test in the case of failed equal variance or normality). Significance within temperature acclimations between individual experimental series, where more than two treatments are compared, was determined using a one-way ANOVA with a Holm–Sidak post-hoc test. Significance within temperature acclimations between individual experimental series, where only two treatments are compared, was determined by a Student's t-test (Mann–Whitney rank sum test in the case of failed equal variance or normality). All values represented as percentages were normalized using an arcsine transformation prior to performing statistical analyses.

3. Results

3.1. Effects of the ILCM on ammonia and urea handling

Degree of coverage of the gills by the ILCM, as assessed by light microscopy, was significantly greater in the 5 °C-fish of all 3 experimental series (Fig. 1). Measurements of coverage index using ESEM (see Fig. 2 for example photos) confirmed this difference. The absolute values of coverage index by ESEM, compared to light microscopy, were significantly lower in 5 °C-fish and slightly lower in 25 °C-fish ($P=0.056$) (Table 1). The relative difference in coverage between each acclimation temperature, however, was similar using light microscopy (1.3-fold difference) and ESEM (1.5-fold difference). No significant differences occurred between experimental series within a given acclimation temperature.

Mean J_{amm} of 25 °C-fish in the whole-body control series was significantly greater than that of the 5 °C-fish (Fig. 3A). Fasting 25 °C-fish for 5 days did not result in a significantly different mean J_{amm} (−1.17 ± 0.12 μmol/g/h; data not shown). J_{amm} was also greater in the 25 °C-fish of the total and divided chamber series than the 5 °C-fish of the same series (Fig. 3A and C). 25 °C-fish did not show

Table 1
Mean coverage indices for control, non-anaesthetized fish as scored using light microscopy or environmental scanning electron microscopy.

		Light microscopy	Environmental scanning electron microscopy
Temperature (°C)	25	0.719 ± .072	0.435 ± .012
	5	0.947 ± .022*	0.688 ± .068*,†

Crosses represent significant differences between scoring methods at a given acclimation temperature. Asterisks represent significant differences between temperature acclimations using the same scoring method. All comparisons were made using unpaired Student's t-tests (or Mann–Whitney rank sum test in the case of failed equal variance or normality).

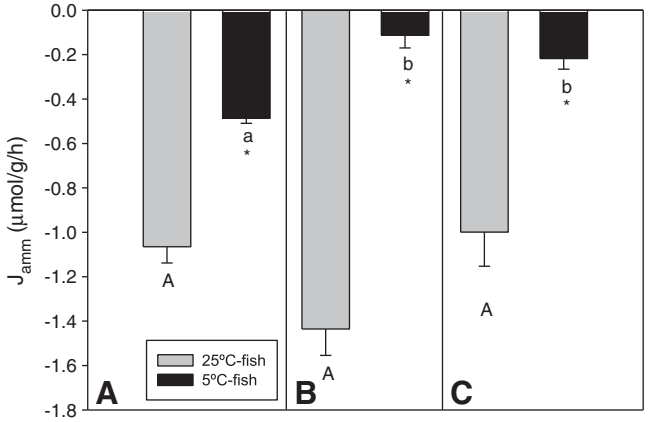

Fig. 3. Mean ammonia excretion (J_{amm}) over a 2-h flux period in 25 °C and 5 °C-fish. A: Whole-body control series ($n=8$). B: Whole-body anaesthetic series ($n=5$–8). C: Total (anterior and posterior) divided chamber series ($n=8$–10). Means sharing the same letter are not significantly different within temperature acclimation (25 °C, upper case letters; 5 °C, lower case letters). Asterisks represent significant differences between 25 °C and 5 °C-fish within each experimental series.

significant differences in mean J_{amm} between experimental series; however, the J_{amm} values of 5 °C-fish were substantially lower in the whole-body anaesthetic and divided chamber series, respectively, in comparison to the control series.

J_{urea} within the whole-body control series was also significantly greater in the 25 °C-fish than in the 5 °C-fish (Fig. 4A) and this same observation was made within the divided chamber series (Fig. 4B). There were no significant differences in J_{urea} within temperature acclimation between the two experimental series. In the whole-body control series, the % total N-excretion as urea-N was greater in 25 °C-fish (29%) than in 5 °C-fish (13%) but this difference was not statistically significant (data not shown; $P=0.052$). The same result was observed in the total divided chamber series (24% and 12% in 25 °C-fish and 5 °C-fish, respectively. $P=0.31$; data not shown).

Terminal plasma T_{amm} was not significantly different between any temperature acclimation or experimental series (Table 2). In the whole-body control series, plasma ammonia clearance rate was about 2-fold higher in 25 °C-fish than in 5 °C-fish, a difference which was not significant ($P=0.066$) (Fig. 5A). In contrast, plasma clearance rates were 33-fold greater in 25 °C-fish than in the 5 °C-fish in the whole-body anaesthetic series (Fig. 5B). 25 °C-fish also displayed an approximately 10-fold greater plasma clearance rate than

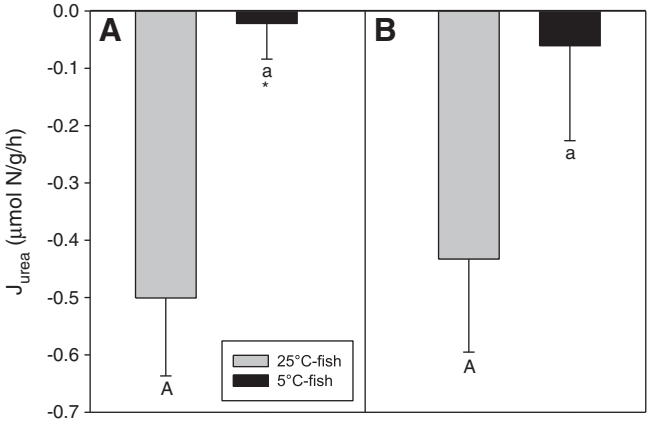

Fig. 4. Mean urea-N excretion (J_{urea}) over a 2-h flux period in 25 °C and 5 °C-fish. A: Whole-body control series ($n=8$). B: Total (anterior and posterior) divided chamber series ($n=6$–8). Means sharing the same letter are not significantly different within temperature acclimation (25 °C, upper case letters; 5 °C, lower case letters). Asterisks represent significant differences between 25 °C and 5 °C-fish within each experimental series.

Table 2
Plasma total ammonia (T_{amm}; μmol/L) in 25 °C and 5 °C-fish in whole-body control, anaesthetic control, divided chamber, and HEA experimental series.

		Experimental series			
		Whole-body control	Whole-body anaesthetic control	Divided chamber	HEA
Temperature (°C)	25	1176.3 ± 275.2	730.5 ± 389.3	1199.7 ± 446.7	1860.8 ± 155.9
	5	968 ± 210.5	968.8 ± 170.4	1829.0 ± 521.2	1634.1 ± 285.0

No significant differences existed between any of the non-HEA groups or between whole-body control and HEA series ($n = 6–8$).

5 °C-fish in the divided chamber series (Fig. 5C). Clearance rates in 25 °C-fish did not vary significantly across experimental series; however, at 5 °C, clearance rates in the whole-body series did vary significantly from both the anaesthetic and the total divided chamber series (Fig. 5).

When anterior J_{amm} was compared to coverage index in both temperature groups within the total divided chamber series, a linear trend emerged ($r^2 = 0.83$; $P < 0.0001$) where fish with a higher degree of coverage (5 °C-fish) showed a markedly lower J_{amm} than those with a lower degree of coverage (25 °C-fish) (Fig. 6A). This linear trend was not seen in plasma ammonia concentrations as no difference in plasma T_{amm} existed between temperature acclimation groups (Table 2). Interestingly, plasma ammonia clearance rates also correlated with ILCM coverage ($r^2 = 0.92$; $P < 0.0001$; Fig. 6B), though this relationship did not fit the linear curve seen in Fig. 6A. Instead, 5 °C-fish all displayed low clearance rates, irrespective of the degree of ILCM coverage, whereas in the 25 °C-fish, plasma clearance of ammonia correlated linearly with ILCM coverage ($r^2 = 0.64$; $P = 0.0109$) (Fig. 6B). J_{urea} did not show a correlation with coverage index ($r^2 = 0.17$, $P = 0.0793$; data not shown).

3.2. The effect of the ILCM on ammonia excretion partitioning

Anterior J_{amm} in 25 °C-fish was approximately 6-fold greater than in 5 °C-fish (Fig. 7A). Posterior J_{amm} was also greater in 25 °C-fish though only by approximately 2-fold (Fig. 7B). Anterior J_{amm} was 11-fold and 4-fold greater than posterior J_{amm} in 25 °C-fish and 5 °C-fish, respectively (Fig. 7).

When taken as a percentage of total J_{amm}, anterior excretion accounted for 90% in 25 °C-fish with posterior excretion accounting for the remaining 10% (Fig. 8A). 5 °C-fish depended significantly more upon posterior routes of excretion, with 74% of J_{amm} from anterior routes, and 26% excretion from posterior routes (Fig. 8B). Anterior to posterior partitioning of J_{urea} was not significantly different between temperature acclimation groups, averaging 95% anterior and 5% posterior (data not shown).

3.3. Effect of the ILCM on ammonia excretion in response to HEA

J_{amm} was reversed in 25 °C-fish exposed to HEA (Fig. 9), representing ammonia uptake. 5 °C-fish, however, maintained excretion during HEA, though at approximately half the rate seen under control conditions (Fig. 9). Plasma T_{amm} in HEA exposed fish was greater than control values for both 25 °C and 5 °C-fish; however, this difference was not significant in either temperature group ($P = 0.072$ and $P = 0.081$ for 25 °C and 5 °C-fish, respectively) (Table 2).

When compared against degree of ILCM coverage, J_{amm} decreased linearly with increasing coverage index ($r^2 = 0.525$; $P = 0.037$), where 25 °C-fish, with the least degree of coverage showed the highest uptake, and 5 °C-fish, with higher degrees of coverage, demonstrated either very small amounts of uptake or excretion against the imposed T_{amm} gradient (Fig. 10).

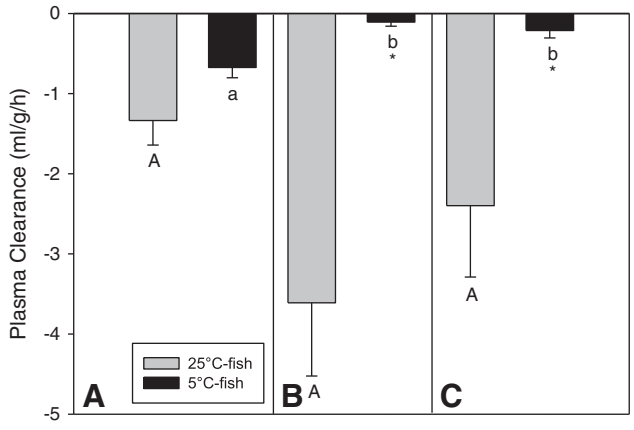

Fig. 5. Mean plasma ammonia clearance rate (mL/g/h) over a 2-h flux period in 25 °C and 5 °C-fish. A: Whole-body control series ($n = 8$). B: Whole-body anaesthetic series ($n = 5–8$). C: Total (anterior and posterior) divided chamber series ($n = 6–8$). Means sharing the same letter are not significantly different within temperature acclimation (25 °C, upper case letters; 5 °C, lower case letters). Asterisks represent significant differences between 25 °C and 5 °C-fish within each experimental series.

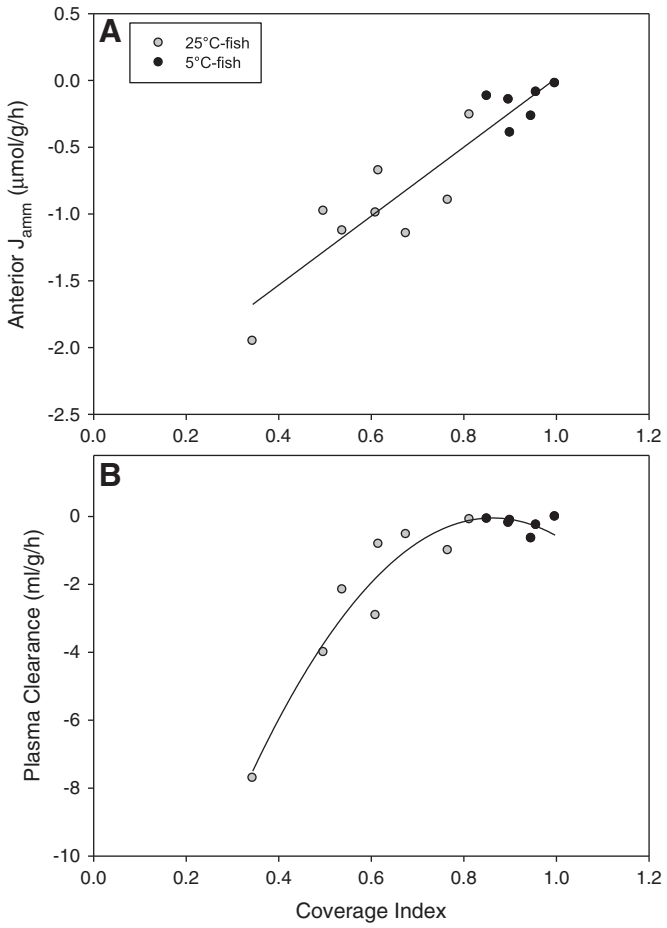

Fig. 6. Relationships of (A) anterior ammonia excretion (J_{amm}) ($r^2 = 0.83$; $P < 0.0001$) vs. coverage index and (B) total (anterior and posterior) plasma clearance rate ($r^2 = 0.92$; $P < 0.0001$) vs. coverage index in 25 °C and 5 °C-fish within the divided chamber series ($n = 6–8$).

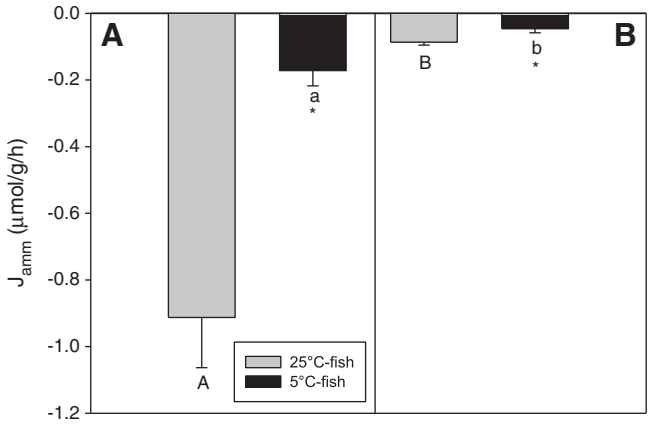

Fig. 7. Partitioning of ammonia excretion (J_{amm}) in 25 °C and 5 °C-fish. A: Anterior J_{amm}. B: Posterior J_{amm}. Means sharing the same letter are not significantly different within temperature acclimation (25 °C, upper case letters; 5 °C, lower case letters). Asterisks represent significant differences between 25 °C and 5 °C-fish within each experimental series ($n=8$–10).

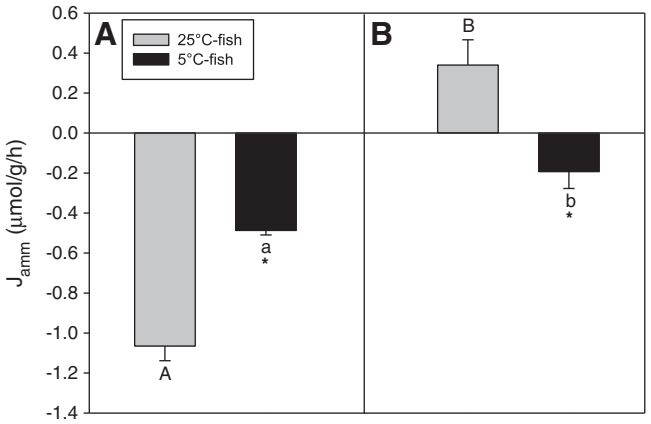

Fig. 9. Mean ammonia excretion (J_{amm}) over a 2-h flux period in 25 °C and 5 °C-fish. A: Whole-body control series ($n=8$). B: Whole-body HEA series ($n=6$–8). Means sharing the same letter are not significantly different within temperature acclimation (25 °C, upper case letters; 5 °C, lower case letters). Asterisks represent significant differences between 25 °C and 5 °C-fish within each experimental series.

4. Discussion

4.1. Overview

In the current study, fish were anaesthetized in order to assess the relative distribution between anterior and posterior J_{amm}. Our control tests demonstrated that anaesthesia alone can apparently reduce J_{amm}, but this observation was only true of 5 °C-fish (Fig. 3). The reason for this effect occurring only for the lower temperature acclimation group is unknown. What is of significance, however, is that no differences occurred between the anaesthetic control group and whole-body divided chamber groups (Fig. 3). We interpret this result to mean that the anaesthetic treatment effectively reduced any stress which may have been associated with this experimental procedure. In the proceeding text, we argue that the difference in J_{amm} between acclimation temperatures appears to be related to the degree of ILCM coverage, regardless of any confounding anaesthetic or temperature effects.

To assess gill coverage, a novel method of scoring gill coverage based on observations made using light microscopy was used. In order to validate this technique, a separate group of fish was acclimated to 25 °C and 5 °C using the same acclimation protocol as all other fish used in the study and gill arches were examined using ESEM (Fig. 2). Though absolute values of coverage index were lower using ESEM than using light microscopy, the relative difference in coverage between temperature acclimation groups remained the same. Thus, it appears that our method of assessing gill coverage using light microscopy, though not as sensitive as ESEM, appears to be a valid technique for determining relative coverage indices between 25 °C-fish and 5 °C-fish. To our knowledge, this is the first time that ESEM has been used for this purpose. An advantage of this new approach, relative to traditional SEM, is its ease – the gill tissue needs only simple fixation prior to visualization. There is no need to sputter-coat the samples, so it is faster, and there is less chance of loss of the intra-lamellar cell mass during processing.

Previous studies on the plastic remodeling of goldfish gills have reported that complete loss of the ILCM occurs at 25 °C in response to hypoxia or an increase in temperature (Sollid et al., 2005; Perry et al., 2010), whereas we were only able to achieve a partial loss of ILCM coverage. However, it is important to note the differences in acclimation regimes between these studies and the study presented here. Sollid et al. (2005) acclimated goldfish for an entire month at 25 °C prior to experimentation. Perry et al. (2010) acclimated their animals to 18 °C for at least a week, subsequently raised the tank temperature by 1 °C per day until 25 °C was reached, and then kept the animals at this temperature for a further 2 weeks prior to experimentation. In both studies, the acclimation regime resulted in a complete loss of the ILCM at 25 °C. In the present study, however, the ILCM was not entirely shed during the 2-week 25 °C acclimation period within any experimental series (Fig. 1). This likely reflects the different

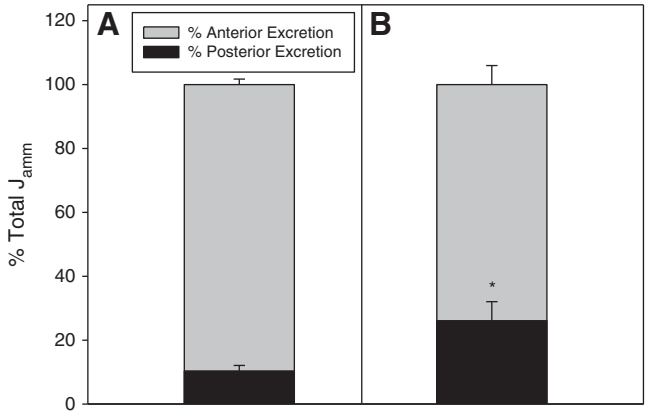

Fig. 8. Relative anterior and posterior ammonia excretion (J_{amm}) in divided chamber series. A: 25 °C-fish ($n=8$). B: 5 °C-fish ($n=6$).

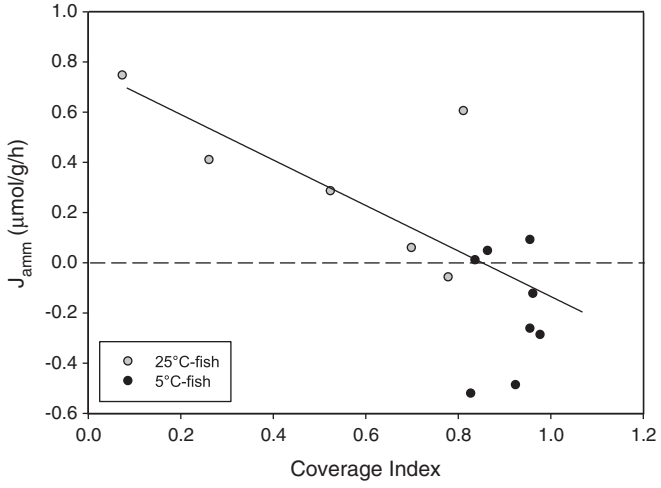

Fig. 10. The relationship of ammonia excretion (J_{amm}) ($r^2=0.525$; $P=0.037$) vs. coverage index in 25 °C and 5 °C-fish exposed to HEA ($n=6$–8).

protocols employed. Time-dependence of ILCM loss has been demonstrated in the goldfish, where exposure to hypoxia at 7 °C for 1 day reduced the ILCM to approximately 69% of original interlamellar area coverage, and an additional 6 days of hypoxia only reduced this value to 39% (Mitrovic et al., 2009). Thus, it is possible that with a different acclimation protocol, total ILCM loss could have been achieved. Nevertheless, a higher degree of coverage was seen in 5 °C-fish (Fig. 1), suggesting that these fish may have been subject to an impedance of branchial ammonia excretion, if the ILCM acts as a diffusive barrier for ammonia movement.

4.2. Effects of the ILCM on net ammonia and urea excretion

In all experimental series, J_{amm} was greater in 25 °C-fish fish than in 5 °C-fish (Fig. 3). Perry et al. (2010), using similar temperature acclimation (7 °C and 25 °C), though considerably larger fish (25 g), did not show differences in J_{amm} between acclimation temperatures under control, unfed conditions. These differences may reflect allometric effects where the smaller fish used in this study, having a greater surface area to volume ratio, would be impacted by temperature changes to a greater degree than larger fish. This group also demonstrated that feeding history had a profound effect upon control J_{amm} in these fish (Perry et al., 2010). Thus, it was initially hypothesized that perhaps the differences in J_{amm} between acclimation temperatures in our study may have been due to a considerable difference in feeding rates between the two temperature groups, as the nutritional demand of 25 °C-fish was much greater than that of 5 °C-fish (see Materials and methods). Fasting 25 °C-fish for an additional 72 h (5 d total), however, did not result in a mean J_{amm} significantly different from that of the 48 h-fasted control group, demonstrating that differences in feeding history likely did not contribute to the observed differences in J_{amm}. The differences in J_{amm} observed between temperature acclimation groups may have also been induced by temperature itself, rather than the ILCM. The connection between temperature increases and resulting increases in ammonia excretion has been seen in a variety of fish species (goldfish: Maetz, 1972; trout: Payan and Matty, 1975; walleye: Cai and Summerfelt, 1992; sturgeon: Gershanovich and Pototskij, 1995). This temperature-dependent increase in ammonia excretion may be at least partly related to an increase in branchial perfusion and branchial ammonia diffusion that can occur during periods of increased temperature (Payan and Matty, 1975). Maetz (1972) also determined that J_{amm} in non-fed fish, being derived solely from metabolic production, displayed a Q_{10} value of 4, suggesting a high dependency upon temperature. The interpretation of this result, common to a variety of teleost species, is that at higher temperatures, nonfed fish exhibit increased aerobic metabolism fueled by the oxidation of proteins (see Wood, 2001 for review). Nevertheless, Maetz (1972) also demonstrated that the diffusive portion of ammonia excretion (that above metabolic excretion), using NH_4Cl plasma loading, had a Q_{10} of 1.9. This lower Q_{10} implies that a portion of the temperature sensitivity in goldfish, specifically, is independent of metabolic production and occurs presumably as a result of changes in branchial permeability.

In the present study, a strong linear correlation ($r^2 = 0.83$; $P < 0.0001$) of branchial (anterior) J_{amm} with the degree of ILCM coverage was seen within the total divided chamber control series (Fig. 6A). Thus, it appears that the observed differences in J_{amm} between temperature acclimation groups are not likely due entirely to temperature-driven changes in respiration or metabolism. This is consistent with the study by Perry et al. (2010) where clearing of intraperitoneal injections of NH_4Cl or NH_4HCO_3 was greater in 25 °C-acclimated than in 7 °C-acclimated goldfish. Moreover, in our 25 °C-fish, plasma ammonia clearance rates also correlated with the degree of ILCM coverage, implying that ILCM presence increases the time it would take the fish to completely clear ammonia from a given volume of plasma. This again suggests that the ILCM may somehow impede the effectiveness of the gills in eliminating ammonia from the body. In 5 °C-fish, however, clearance rates were independent of ILCM coverage (Fig. 6B). Perhaps at this acclimation temperature, clearance rates are more dependent upon possible temperature-induced changes in branchial permeability and diffusion, rather than upon the degree of ILCM coverage.

Plasma T_{amm} values did not fit with our initial hypothesis of the ILCM acting as barrier to ammonia excretion (Table 2). We then speculated that perhaps 5 °C-fish were able to convert ammonia to urea in order to avoid toxicity as has been demonstrated in zebrafish exposed to HEA (Braun et al., 2009). However, J_{urea} was also much lower in 5 °C-fish (Fig. 4) and did not correlate with ILCM coverage. This difference was likely due to the aforementioned temperature-driven physiological effects on respiration and protein catabolism. Moreover, % total N-excretion as urea-N was not significantly different between temperature acclimation groups which may imply that 5 °C-fish do not selectively excrete one nitrogenous waste product or another in response to increased ILCM presence. Unfortunately, in light of the small volumes of blood plasma available, it was not possible to measure plasma urea concentration. Therefore, the accumulation of urea within the plasma or tissue to avoid toxicity may still be a possible mechanism to avoid potential ammonia toxicity.

4.3. The effect of the ILCM on ammonia partitioning

Homer Smith (1929), using carp and goldfish, was the first to demonstrate that the majority of ammonia excretion (94%) occurred branchially. This anterior–posterior partitioning is in agreement with the 25 °C-fish of this study (Figs. 7 and 8). 5 °C-fish, however, relied more on posterior routes of excretion on a relative basis (Fig. 8), suggesting that ILCM presence, impeding branchial excretion, forces these fish to shunt ammonia excretion to these extra-branchial routes. In this study, we were unable to determine which posterior routes would be most utilized by these fish. Interestingly, recent studies on the cutaneous expression of Rh genes in rainbow trout (*Oncorhynchus mykiss*), in response to HEA exposure, suggest that the skin may serve as a potential extra-branchial route of ammonia excretion (Nawata et al., 2007; Nawata and Wood, 2009). Note that this trend of 5 °C-fish relying more upon posterior routes of excretion was not observed for urea excretion.

4.4. The effects of the ILCM on ammonia excretion in response to HEA

The reversal and subsequent re-establishment of J_{amm} is a consistent response to HEA exposure in many freshwater fish (Wilson et al., 1994; Nawata et al., 2007; Braun et al., 2009; Perry et al., 2010; Zimmer et al., 2010). In this study, 25 °C-fish exhibited the characteristic reversal of excretion (Fig. 9) over the 2-h flux period, in accord with these previous studies. However, the ability of 5 °C-fish to maintain excretion, albeit to a lesser degree than under control conditions, was notable (Fig. 9). This is consistent with the notion that the ILCM may act as a diffusive barrier for ammonia, in this case preventing in ammonia uptake in 5 °C-fish. The relatively lower degree of ILCM coverage in 25 °C-fish allowed for the uptake of ammonia during HEA exposure. The notion of the ILCM preventing ammonia accumulation during HEA could not, however, be confirmed by plasma T_{amm} values. Both acclimation groups demonstrated a tendency for increased plasma T_{amm} during HEA exposure but this was not significant (Table 2). Note, however, that since only very small volumes of plasma were obtained, we had to dilute our samples substantially to be able to measure T_{amm}, so these data should be interpreted with caution. Perhaps with a longer exposure, more pronounced differences in these values would have been observed, though 2 h was sufficient to elevate plasma T_{amm} in juvenile rainbow trout exposed to the same concentration of ammonia (Zimmer et al., 2010). Finally, J_{amm} in this

series also correlated with ILCM coverage (Fig. 10), though in the opposite direction than under non-HEA conditions (Fig. 6A), providing further evidence that ILCM may impede ammonia movement and that, furthermore, this impedance appears to be bidirectional.

In conclusion, there is an apparent direct effect of the ILCM on branchial (anterior) J_{amm}. We have demonstrated that J_{amm} in control and HEA conditions can be correlated to the degree of ILCM coverage. This suggests that the ILCM may, in fact, act as a barrier for ammonia transport and that fish may undergo physiological changes in order to compensate for a reduction in branchial J_{amm}. Though 5 °C-fish in the present study did not demonstrate a shift to rely more upon urea excretion to excrete nitrogenous waste, we cannot rule out the potential of these fish storing urea in the plasma or tissues to avoid ammonia toxicity. Furthermore, 5 °C-fish demonstrated a greater relative proportion of posterior J_{amm} than did 25 °C-fish. Thus, our two initial hypotheses (reduced whole-body J_{amm} and increased reliance on extra-branchial excretion at 5 °C) are supported. The present research sheds light on the physiological strategies of this unique fish species in response to branchial compensations which are made to adjust oxygen uptake and ion loss rates. We suggest a need for future studies to investigate the effects of hypoxia-induced loss of the ILCM on J_{amm} and if this would also lead to changes in nitrogen handling (i.e., urea excretion/storage) or changes in extra-branchial J_{amm}. Finally, in light of recent research, it will be interesting to determine whether or not these fish express Rh proteins in the skin and if cutaneous surfaces are utilized for ammonia excretion during periods of reduced branchial excretion.

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