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EXCRETION AND DISTRIBUTION OF AMMONIA AND THE INFLUENCE OF BOUNDARY LAYER ACIDIFICATION IN EMBRYONIC RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

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Summary

This study examined ammonia excretion by embryos of the rainbow trout (*Oncorhynchus mykiss*). The distribution of ammonia in relation to the H^+ distribution and electrical potential was determined. The influence of the pH of the unstirred layer (USL) of water next to the external surface of the embryo was also assessed. Eyed-up embryos (35–40 days post-fertilization) were exposed to various external water conditions [pH 6.0, pH 10.0, 1.6 mmol l^{-1} NaCl, 0.0 mmol l^{-1} NaCl, 0.2 mmol l^{-1} NH_4Cl, 2.5 mmol l^{-1} borax buffer ($Na_2B_4O_7{\cdot}10H_2O$), 2.5 mmol l^{-1} Hepes, 0.1 mmol l^{-1} amiloride] for 30 min and ammonia excretion rates, ammonia concentration in the perivitelline fluid (PVF) and yolk, and the pH of the PVF, yolk and USL were measured. The rate of ammonia excretion was dependent, in part, on the partial pressure gradient of NH_3 (ΔP_{NH_3}) from the PVF to the USL. Exposure to water of pH 6 increased, whereas NH_4Cl or pH 10 exposure decreased, ammonia excretion rates. Elevated external Na^+ levels also influenced the rate of ammonia excretion, but neither Na^+-free water nor amiloride had any effect. The distribution of ammonia between the PVF and USL was dependent on the H^+ distribution, but ammonia was distributed according to the electrical potential between the PVF and yolk. The USL was 0.32 pH units more acidic than the bulk water. Addition of buffer to the external water eliminated the acid USL and decreased ammonia excretion rates. We conclude that rainbow trout embryos excrete ammonia primarily as NH_3, but when external Na^+ levels are elevated, ammonia excretion may be independent of the P_{NH_3} gradient. The acidic USL next to the chorion probably facilitates NH_3 diffusion by maintaining the ΔP_{NH_3} through the conversion of NH_3 to NH_4^+ upon entry into the USL.

Key words: ammonia transport, ammonia excretion, yolk, perivitelline fluid, pH, amiloride, acid unstirred layer, electrical potential, rainbow trout, *Oncorhynchus mykiss*.

Introduction

The primary nutrients in oviparous teleost embryos are the yolk proteins (Kamler, 1992) and, consequently, nitrogenous wastes such as ammonia, urea and uric acid must be stored, recycled or excreted during embryonic development. The embryo consists of the fertilized egg containing the developing embryonic tissue and the attached yolk sac, a surrounding layer of perivitelline fluid (PVF), and an encasing acellular coat or chorion. Ammonia and urea accumulate steadily throughout embryonic development, but only ammonia excretion was detected in trout embryos prior to hatching (Smith, 1947; Rice and Stokes, 1974; Wright *et al.* 1995). Relatively low concentrations of uric acid were found within the embryos and no uric acid excretion was detected (Rice and Stokes, 1974).

There is no information in the literature, to our knowledge, on ammonia transport and distribution within the fish embryo. Ammonia movement across most biological membranes is primarily by NH_3 diffusion, down the partial pressure gradient (ΔP_{NH_3}). With a pK of approximately 9.5, P_{NH_3} will be higher in more alkaline tissue compartments, and NH_3 will therefore diffuse into more acidic compartments. The traditional 'diffusion trapping model' described by Pitts (1973) predicts that total ammonia concentration will be higher in more acidic tissues because NH_3 which diffuses in becomes protonated and trapped as NH_4^+. This model assumes that NH_4^+ is essentially impermeable. Some teleost tissues, including rainbow trout white muscle (Wright *et al.* 1988*b*; Wright and Wood, 1988) and lemon sole (*Parophrys vetulus*) white muscle, heart and brain (Wright *et al.* 1988*a*), are permeable to both NH_3 and NH_4^+. Whether NH_4^+ can also diffuse across either the yolk-sac epithelium or the chorion is unknown. It is possible, therefore, that the partitioning of ammonia within the embryo and across the chorion depends on both the electrochemical NH_4^+ gradient and the pH-dependent P_{NH_3} gradient.

In adult rainbow trout, ammonia excretion is influenced by

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the pH of the external bulk water (e.g. McDonald and Wood, 1981; Wright and Wood, 1985; Wilkie and Wood, 1991). There is an acid-water boundary layer next to the gills (Wright *et al.* 1986; Playle and Wood, 1989) which is maintained by CO_2 and H^+ excretion (Lin and Randall, 1990, 1991). Wright *et al.* (1989) demonstrated that the acid-water boundary layer enhanced blood-to-water NH_3 diffusion gradients and facilitated ammonia excretion in rainbow trout. Rombough (1988) proposed that an unstirred boundary layer (USL) is present around salmonid embryos, but whether it is acidic and influences ammonia excretion is unknown.

This study was designed to test the following hypotheses. (1) The primary mechanism of ammonia excretion from the embryo to the external environment is by diffusion of NH_3 down the partial pressure gradient. (2) The distribution of ammonia between the yolk and the PVF and between the PVF and the external water is dependent on the pH gradient between these fluid compartments. (3) The pH of the USL adjacent to the embryo is acidic relative to the bulk water.

Embryos were exposed to various external water conditions [pH 6.0, pH 10, 1.6 mmol l^{-1} NaCl, 0.0 mmol l^{-1} NaCl, 0.2 mmol l^{-1} NH_4Cl, 2.5 mmol l^{-1} borax buffer ($Na_2B_4O_7{\cdot}10H_2O$), 2.5 mmol l^{-1} Hepes and 0.1 mmol l^{-1} amiloride] for 30 min. Ammonia concentrations and excretion rates to the environment were measured. The pH of the PVF, yolk and USL were determined using microelectrodes.

Materials and methods

Animals

Eyed-up rainbow trout [*Oncorhynchus mykiss* (Walbaum)] embryos of similar size (35–40 days post-fertilization, mass 0.10±0.00 g, mean ± S.E.M.) from 4-year-old female trout were purchased from Blue Spring Trout hatchery (Hanover, Ontario, Canada), Alma Research Station (Alma, Ontario, Canada) or Rainbow Springs Trout hatchery (Thamesford, Ontario, Canada). For the experiments performed in Guelph, the embryos were maintained in recirculating well water (total hardness=411 mg l^{-1} as $CaCO_3$, $[Ca^{2+}]$=2.6 mequiv l^{-1}, $[Cl^-]$=1.47 mequiv l^{-1}, $[Mg^{2+}]$=1.49 mequiv l^{-1}, $[K^+]$= 0.06 mequiv l^{-1}, $[Na^+]$=1.05 mequiv l^{-1}) in an incubation tray (Heath) at 8 °C. For the experiments performed in Hamilton, the embryos were maintained at 8 °C in shallow plastic containers supplied with recirculated aerated dechlorinated tapwater (total hardness=100 mg l^{-1} as $CaCO_3$, $[Ca^{2+}]$= 1.0 mequiv l^{-1}, $[Cl^-]$=0.75 mequiv l^{-1}, $[Mg^{2+}]$=0.3 mequiv l^{-1}, $[K^+]$=0.03 mequiv l^{-1}, $[Na^+]$=0.6 mequiv l^{-1}). On the day of the experiment, 50–75 embryos from each female were transported from the holding facility to the laboratory by placement in one of four 1 l beakers containing aerated water (8 °C).

Experimental treatments

Separate groups of embryos were exposed for 30 min (8 °C) to one of the following conditions: (1) control water 1 (Guelph) at pH 8.3; (2) elevated external [ammonia] (0.2 mmol l^{-1} NH_4Cl, Guelph) at pH 8.3; (3) control water 2 (Hamilton) at pH 8.3; (4) low water pH of 6.0 (pH adjusted with 0.2 mol l^{-1} H_2SO_4, Hamilton); (5) elevated external Na^+ concentration (1.0 mmol l^{-1} NaCl was added to give a final calculated water Na^+ concentration of 1.6 mmol l^{-1}, Hamilton) at pH 8.3; (6) buffered water I [2.5 mmol l^{-1} borax ($Na_2B_4O_7{\cdot}10H_2O$), Hamilton] at pH 8.3; (7) control water 3 (Guelph) at pH 8.3; (8) elevated water pH of 10.0 (pH adjusted with 0.1 mol l^{-1} KOH; Guelph); (9) control water 4 (Guelph) at pH 7.8; (10) buffered water II (2.5 mmol l^{-1} Hepes; Guelph) at pH 7.8; (11) control water 5 (formulated water: 0.6 mmol l^{-1} NaCl, 0.3 mmol l^{-1} $MgCl_2$, 0.2 mmol l^{-1} $KHCO_3$, 1.6 mmol l^{-1} $CaSO_4$) at pH 8.3; (12) Na^+-free water (0.6 mmol l^{-1} choline chloride, 0.3 mmol l^{-1} $MgCl_2$, 0.2 mmol l^{-1} $KHCO_3$, 1.6 mmol l^{-1} $CaSO_4$) at pH 8.3; (13) control water 6 (Guelph) at pH 8.1; and (14) amiloride (0.1 mmol l^{-1}, Guelph) at pH 7.9.

The water for each treatment was aerated vigorously for a minimum of 4 h before each experiment. For treatments 1–6, four sets of embryos were used in each experiment, each set of embryos produced by an individual 4-year-old female. For treatments 7–14, four subsets of embryos were used from a single 4-year-old female. The variability between embryos from different females was found to be similar to the variability between subsets of embryos from the same female. Consequently, in the ammonia excretion measurements for experiments 7–14 and for pH measurements from the boundary layer experiments, embryos were derived from one female but divided into four groups, where N is the number of repetitions. Treatments 7–14 were carried out after analysis of the results of treatments 1–6 and in order to study the mechanisms of ammonia excretion across the chorion. Consequently, in experiments 7–14, only ammonia excretion to the external water was measured.

Ammonia excretion to the external water

Ammonia excretion was measured over the 30 min experimental treatment in four groups of 20 embryos placed in small glass beakers (40 ml) containing 8 ml of water (8 °C). Water was aerated for the duration of the experiment. Samples (8 ml) were collected at the start from the bulk water container and end of the experiment and immediately frozen at –20 °C for later determination of ammonia concentrations. The embryos were blotted dry and the wet mass was recorded. Excretion rates (in μmol-N g^{-1} h^{-1}) were calculated as the difference in ammonia concentration (0–30 min) (in μmol-N l^{-1}) multiplied by the volume of the chamber (in l) divided by the mass of tissue (in g) and the time period (0.5 h).

Collection of PVF and yolk samples

In a separate group of embryos exposed to the same experimental treatments, PVF and yolk samples were collected. At the end of the experiment, embryos were removed, blotted dry and then submerged in a Petri dish filled with mineral oil. PVF was collected under a dissecting microscope by making a small incision in the chorion of the embryo under oil with a 21 gauge syringe needle and carefully aspirating the resulting drop of PVF using a glass micropipette,

drawn over a flame from a 100 µl disposable capillary tube. The fluid was then transferred to a 0.5 ml vial and was immediately frozen on dry ice. Samples from 20–40 embryos from the same female were pooled in a single vial to ensure a sufficient sample volume for ammonia analysis. Yolk samples were collected by holding the embryo in a pair of tweezers and then squeezing the embryo so that the yolk sac was in direct contact with the chorion along the bottom of the embryo. The embryo was then punctured under oil and with a 21 gauge syringe needle, to release the yolk, leaving the developing tissue and PVF still in the chorion. The yolk sample was then aspirated with a glass micropipette and placed in a separate 0.5 ml vial and immediately frozen on dry ice. For yolk ammonia measurements, samples from 3–6 embryos were pooled. The PVF and yolk samples were frozen at –80 °C and analyzed for ammonia within 1 week.

Water, PVF and yolk ammonia analysis

Water samples were analyzed for ammonia using the colorimetric assay described by Anderson *et al.* (1987). For PVF and yolk analysis, samples were thawed and immediately deproteinized with one and two volumes of ice-cold 8 % $HClO_4$, respectively. Samples were centrifuged at 12 000 ***g*** for 10 min at 4 °C. The supernatant was neutralized with saturated $KHCO_3$ and then centrifuged at room temperature (22 °C) at 12 000 ***g*** for 4 min. Ammonia concentration was determined on the neutralized supernatant (Sigma diagnostic kit 171-C).

Measurement of pH of the PVF and yolk

The construction and use of pH microelectrodes based on the neutral carrier tridodecylamine (H^+ ionophore I, cocktail B, Fluka Chemical Corp., Ronkonkoma, NY, USA) have been described elsewhere (Maddrell *et al.* 1993; O'Donnell and Maddrell, 1995).

The pH of the PVF and yolk samples were measured under paraffin oil to prevent evaporation of the samples. The embryos were held with microforceps and punctured using a fine syringe needle (25 gauge). The pH and reference microelectrodes were positioned in the samples, and the electrical potential recorded. The pH was then calculated from the difference in electrical potential recorded between the sample and a calibration solution of known pH. Calibration solutions consisted of physiological saline adjusted to two pH values differing by 1 unit. Preliminary experiments showed that the pH microelectrodes were not sensitive to changes in ionic strength; the microelectrode potential changed by less than 1 mV when the electrode was transferred between physiological saline and fresh water buffered with 1 mmol l^{-1} Hepes at the same pH.

Microelectrode measurements of the pH of intra- and extracellular fluids are typically reported to 0.01 pH unit because the reproducibility of such measurements is typically less than 0.01 pH unit (Amman, 1986).

Measurement of pH and [NH_4^+] of the boundary layer

Double-barrelled pH and NH_4^+-selective microelectrodes were constructed from unfilamented double-barrelled borosilicate glass tubing (1.2 mm o.d., 0.68 mm i.d.; A-M Systems, Inc., Everett, WA, USA). Tubing was acid-washed, dried on a hot plate at 200 °C and pulled to tip diameters of less than 1 µm. The micropipettes were then silanized as for single-barrelled electrodes. After silanization, one barrel was filled with the H^+ ionophore and the other with ammonium ionophore based on 75 % nonactin/25 % monactin (Ammonium ionophore I, cocktail A, Fluka). Backfilling solutions for pH and NH_4^+-selective barrels were 100 mmol l^{-1} NaCl/100 mmol l^{-1} sodium citrate (pH 6.0) and 1 mol l^{-1} NH_4Cl, respectively.

The pH and NH_4^+ concentrations of the water in the unstirred layer (USL) were measured within 10–20 µm of the surface of the embryo, or in the bulk water (BW) at least 1 cm distant from the embryo. The measurements were achieved by measuring the potentials of both barrels with respect to a reference electrode filled with 3 mol l^{-1} KCl placed in the water at more than 1 cm distance from the embryo. Because distilled water typically contains approximately 10^{-5} mol l^{-1} ammonia, ammonia standards in the micromolar range were prepared by first passing distilled water through Dowex-50 (Sigma) ion-exchange resin to remove ammonia, than adding back known levels of ammonia and salts (0.5 mmol l^{-1} $CaCl_2$ and 0.5 mmol l^{-1} Na_2CO_3). The concentrations of $CaCl_2$ and Na_2CO_3 were chosen to mimic Hamilton tap water (see above).

The pH and NH_4^+ concentration in the USL and BW were measured as stated above in the control, pH 6.0 and buffer I experiments only. Measured total ammonia concentrations in the BW were used to calculate NH_4^+ and P_{NH_3} in the NH_4Cl and NaCl experiments.

Measurement of electrical potentials

Double-barrelled micropipettes were pulled from thick septum theta glass (TST150, WPI, Sarasota, FL, USA). When both barrels were filled with 3 mol l^{-1} KCl, the resulting microelectrodes had tip resistances of 20–40 MΩ. The natural spear point and thick central septum of these microelectrodes facilitated impalement of the chorion. One barrel was connected to an electrometer (M707, WPI) through a chlorided silver wire. For measurement of perivitelline potentials, an embryo was first positioned under water in a hemispherical depression in a Sylgard-lined Petri dish. The chorion was then impaled by a microelectrode mounted on a hydraulic micromanipulator (Narishige, Tokyo) and then a second microelectrode was positioned in the water to serve as a reference. For measurement of vitelline potentials, a slit was made in the chorion with a 26 gauge syringe needle. One microelectrode was advanced into the PVF to serve as a reference, and the other was advanced across the yolk sac or vitelline membrane. All potentials were recorded on a computerized data acquisition system (Axotape, Burlingame, CA, USA).

Evaluation of pK

The accuracy of the ammonia gradient calculations between different compartments in the embryo depends largely on the

precision of the pH measurements and the use of the correct physical constants (Cameron and Heisler, 1983; Wright and Wood, 1985). Very little is known about the properties of PVF and yolk solutions in embryonic rainbow trout; therefore, a number of estimations were made on the basis of the available literature. We assumed that the composition of the PVF of rainbow trout embryos was similar to that of the Atlantic salmon *Salmo salar* [58–71 % water, 20–25 % protein, 5–12 % lipid and 1.0–1.7 % carbohydrate, with an osmolarity of approximately 30 mosmol l^{-1} ([NaCl]=3–5 mmol l^{-1}); Rudy and Potts, 1969; Eddy, 1974; Hamor and Garside, 1977; Shephard and McWilliams, 1989] as both these salmonid embryos develop in fresh water. To our knowledge, there have been no published values for the constituents of trout PVF.

To determine the apparent pK of ammonia (pK'_{amm}) in the PVF, a NaCl equivalent value of 10 mmol l^{-1} was used. This approximate value was based on the findings of Cameron and Heisler (1983) who reported that, in terms of pK'_{amm}, trout plasma behaved like a solution with twice the osmolarity that was actually measured (owing to the presence of dissolved ions, proteins and fatty components).

For the yolk, a value of 370 mmol l^{-1} NaCl equivalents was used to determine pK'_{amm}. Although the yolk contains a higher level of protein and lipid than trout plasma, the yolk of an ovulated egg is in osmotic equilibrium with the ovarian fluid, which has a similar osmotic concentration to the blood plasma (Hirano *et al.* 1978; Riis-Vestergaard, 1982). A further assumption was made that the osmolarity of the yolk remained fairly constant once the embryos were shed into the water because the yolk membrane is highly impermeable to water and H^+ (Potts and Rudy, 1969). Moreover, the percentage water content of the whole embryo did not change in rainbow trout embryos from 8 days after fertilization to hatching at 11–13 °C (Wright *et al.* 1995).

Evaluation of ammonia solubility coefficients

Calculation of P_{NH_3} from NH_3 concentration requires the NH_3 solubility coefficient (α_{NH_3}), which depends on both temperature and ionic strength (Cameron and Heisler, 1983). To our knowledge, there are no data available for α_{NH_3} values in other fish fluids such as PVF or yolk. It was assumed that PVF and yolk NH_3 solubility coefficients (α_{NH_3}) were similar to those of rainbow trout plasma.

Calculations

NH_4^+ (mmol l^{-1}) concentrations and the partial pressure of NH_3 (P_{NH_3} in nmHg) were calculated as outlined by Wright and Wood (1985). Gradients (ΔP_{NH_3} and $\Delta P_{NH_4^+}$) between tissue compartments were then calculated as the difference in concentration or partial pressure between different compartments.

To determine whether ammonia was distributed according to the H^+ distribution, it was assumed that NH_3 concentration was equal in all compartments and, therefore, the P_{NH_3} in each compartment was set to the PVF P_{NH_3}. A predicted total ammonia concentration (T_{amm}) was calculated for each compartment by manipulation of the Henderson–Hasselbalch equation, using the measured pH for each compartment:

$$\text{predicted } T_{amm} = [NH_3] + \{[NH_3]/\text{antilog}(\text{measured pH} - \text{pK})\} . \quad (1)$$

The predicted T_{amm} was then compared with the measured T_{amm} for each compartment.

To determine whether the distribution of ammonia between the USL, PVF and yolk could be a function of the electrical potential across the yolk-sac membrane or chorion, and in agreement with the measured potentials, the ammonia equilibrium potential $E_{NH_4^+}$ was calculated using the Nernst equation:

$$E_{NH_4^+} = \boldsymbol{R}T/zF \times \ln([NH_4^+]_a/[NH_4^+]_b) , \quad (2)$$

where $\boldsymbol{R}$ is the gas constant (8.314 V C K^{-1} mol^{-1}), T is the absolute temperature (K), z is the charge on the solute, F is the Faraday constant (96 500 C mol^{-1}) and a and b represent the USL and the PVF and yolk, respectively.

Statistics

Data are presented as means ± 1 S.E.M. In treatments 1–6, N=4 represents pooled measurements on embryos from four separate females, and in treatments 7–14, N is the number of repetitions. Student's unpaired t-tests ($P \leqslant 0.05$) were used to evaluate the significance of differences between means. A one-way analysis of variance (ANOVA) was performed to evaluate the difference between means for treatments where more than two treatments were compared with the same control treatment (i.e. control 2 *versus* pH 6.0, 1.6 mmol l^{-1} NaCl and 2.5 mmol l^{-1} borax buffer).

Results

pH of the unstirred water layer and internal tissue compartments

Under control conditions, the pH of the unstirred layer (USL) around the embryo was, on average (N=7), 0.32 pH units acid to the bulk water pH (8.3). The pH of the PVF was approximately 7.17, whereas the yolk pH was considerably more acidic at 6.35–6.48 (Table 1).

Exposure to NH_4Cl resulted in a slight, but significant, decrease in the pH of the PVF, but no change in yolk pH (Fig. 1A). On exposure to an acidic (pH 6.0) external environment, the pH of the BW, USL and PVF were reduced compared with the control value, whereas the pH of the yolk was unchanged (Fig. 2). Elevated external [NaCl] did not change PVF pH, but resulted in a significant increase in yolk pH (Fig. 3A). The addition of borax buffer to the external water effectively eliminated the acid boundary layer, did not change PVF pH, but resulted in a significant elevation of yolk pH (Fig. 4A).

Partitioning of ammonia

Total ammonia concentration (T_{amm}) and NH_4^+ concentration were 4–5 times higher in the yolk than in the

Table 1. *A comparison of pH and measured and predicted total ammonia concentrations in the yolk, perivitelline fluid and unstirred layer based on the* P_{NH_3} *value of the perivitelline fluid*

	Measured				Predicted	Measured		Predicted
	pH_{PVF}	pH_{yolk}	T_{amm} PVF (mmol l^{-1})	T_{amm} yolk (mmol l^{-1})	T_{amm} yolk (mmol l^{-1})	pH_{USL}	T_{amm} USL (mmol l^{-1})	T_{amm} USL (mmol l^{-1})
Control 1	7.18±0.03	6.48±0.04	0.48±0.03	3.34±0.21	3.71±0.21	7.98±0.02	0.05±0.01	0.07±0.00
Control 2	7.17±0.02	6.35±0.04	0.41±0.04	1.87±0.11	4.15±0.36*	7.98±0.02	0.05±0.01	0.06±0.01

Values are means ± S.E.M., N=4, except for pH_{USL} where N=7.
T_{amm}, total ammonia concentration; PVF, perivitelline fluid; USL, unstirred layer; P_{NH_3}, partial pressure of ammonia.
*Significantly different from measured value $P \leqslant 0.05$.
Bulk water pH in control 1 and 2 was 8.3±0.1; see Materials and methods for further details.

PVF (Figs 1B, 2B and Figs 1C, 2C, respectively). A positive concentration gradient existed for NH_4^+ from the yolk to the PVF and from the PVF to the USL (Figs 1C, 2C). In control 1 embryos, there was no difference between P_{NH_3} in the PVF and yolk (Fig. 1D), whereas a positive P_{NH_3} gradient from the PVF to the yolk was present in control 2 embryos (Fig. 2D). There were no significant differences between P_{NH_3} levels in the PVF, USL or BW for either control 1 or 2 embryos (Figs 1D, 2D; note the difference beween axis scales).

Predicted yolk T_{amm} was not significantly different from measured yolk T_{amm} in the control 1 group, but was significantly greater in the control 2 experiment (Table 1). In both control experiments, predicted USL T_{amm} values were not significantly different from measured values (Table 1).

Calculated $E_{NH_4^+}$ values were not significantly different from measured membrane potential (E_M) across the yolk-sac epithelium (yolk–PVF). However, calculated $E_{NH_4^+}$ values differed significantly from measured E_M values across the chorion (PVF–USL) (Table 2).

Influence of external water conditions on ammonia excretion

Under control conditions, ammonia excretion rate ranged between 0.10 and 0.13 μmol-N g^{-1} h^{-1} (Figs 1E, 2E). High external ammonia levels eliminated, and in fact reversed, ammonia excretion rates (Fig. 1E). There was a significant increase in the PVF T_{amm} and [NH_4^+], but not in P_{NH_3} (Fig. 1B–D). Although NH_4^+ levels in the USL and BW were not directly measured in this experiment, the amount of NH_3 and NH_4^+ in the BW was calculated using the measured pH and total ammonia levels. The resulting calculations demonstrated a large positive BW-to-PVF P_{NH_3} gradient (Fig. 1D), but T_{amm} in the BW was lower than in either the yolk or the PVF (Fig. 1B).

Ammonia excretion rate increased twofold in embryos exposed to pH 6.0 water (Fig. 2E). Exposure of the embryo to a low pH significantly decreased T_{amm} and the NH_4^+ concentrations of the yolk (Fig. 2B,C). There were no changes, however, in the directions of the NH_4^+ gradients (Fig. 2C). At water pH 6.0, P_{NH_3} values decreased significantly in the PVF, USL and BW (Fig. 2D). Consequently, the P_{NH_3} gradient was reversed; that is, from yolk to PVF and from PVF to USL.

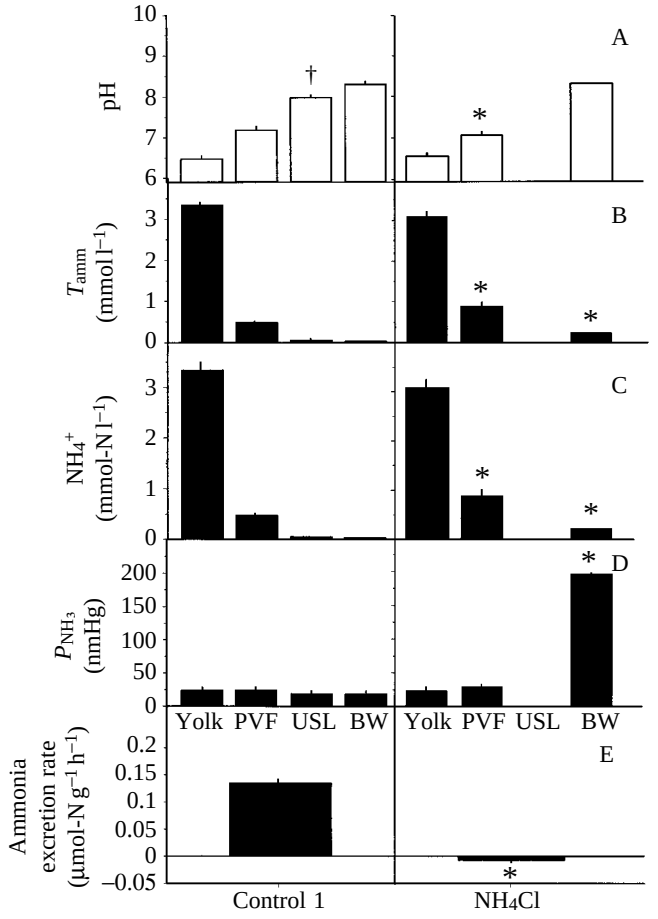

Fig. 1. A comparison between rainbow trout embryos exposed to control water 1 (see Materials amd methods) of pH 8.3 or elevated external ammonia levels (0.2 mmol l^{-1} NH_4Cl, pH 8.3). (A) pH; (B) total ammonia-N concentration (T_{amm}); (C) [NH_4^+]; (D) partial pressure of NH_3 (P_{NH_3}); (E) ammonia excretion rates. Asterisks represent significant differences ($P \leqslant 0.05$) between control and experimental values for the same experiment. Values are given as the mean + 1 S.E.M. (N=4). Daggers represent significant differences ($P \leqslant 0.05$) between the USL and BW pH within the control experiment. PVF, perivitelline fluid; USL, unstirred layer; BW, bulk water. The pH and ammonia concentrations in the USL were not measured for the elevated ammonia treatment. P_{NH_3} and [NH_4^+] in the BW were calculated from measured total ammonia concentrations. See Materials and methods for details.

Table 2. *Measured membrane potential and calculated ammonium equilibrium potential between the unstirred layer and the perivitelline fluid and the perivitelline fluid and the yolk*

	Measured E_M PVF–USL (mV)	Calculated $E_{NH_4^+}$ PVF–USL (mV)	Measured E_M Yolk–PVF (mV)	Calculated $E_{NH_4^+}$ Yolk–PVF (mV)
Control 1	−35.8±1.4	−53.9±2.3*	−41.8±4.8	−47.1±0.9
Control 2	−35.8±1.4	−49.9±2.3*	−41.8±4.8	−37.2±2.1
Mean	−35.8	−51.9	−41.8	−42.0

*Significantly different from measured E_m $P \leq 0.05$.

USL, unstirred layer; PVF, perivitelline fluid; E_M, membrane potential; $E_{NH_4^+}$, ammonium equilibrium potential.

See Materials and methods for details of control water composition.

Exposure of the embryos to elevated NaCl levels increased ammonia excretion rates twofold (Fig. 3E). Elevated [NaCl] did not significantly affect T_{amm} (Fig. 3B) or NH_4^+ level (Fig. 3C). There was, however, a significant increase in the P_{NH_3} value in the yolk (Fig. 3D).

Embryos exposed to buffered water I (2.5 mmol l^{-1} $Na_2B_4O_7{\cdot}10H_2O$) showed a significant increase in ammonia excretion rate (Fig. 4E) and a significant decrease in T_{amm} and the NH_4^+ concentrations of the yolk (Fig. 4B,C). There was no change in the direction of the NH_4^+ gradient (Fig. 4C).

At water pH 10, ammonia excretion rates were significantly reduced compared with control values (−63 %; Fig. 5A). Exposure to buffered water II (2.5 mmol l^{-1} Hepes) reduced ammonia excretion rate by 50 % (Fig. 5B). Exposing the embryos to Na^+-free water (Fig. 5C) or to 0.1 mmol l^{-1} amiloride (Fig. 5D) did not significantly alter ammonia excretion rates in intact embryos. We repeated the amiloride experiment on embryos in which a small hole had been made in the chorion to allow for more rapid exchange between the PVF and BW. As in intact embryos, 0.1 mmol l^{-1} amiloride had no significant effect on ammonia excretion rate (control 0.056±0.004 μmol-N g^{-1} h^{-1} *versus* amiloride 0.051±0.003 μmol-N g^{-1} h^{-1}).

Discussion

An acidic unstirred water layer surrounds the trout embryo

The pH of the unstirred boundary layer around teleost embryos has not been determined previously. The existence of an acidic unstirred layer next to the chorion (0.32 pH units lower than the bulk water) is similar to that estimated for adult rainbow trout gills (Wright *et al.* 1986; Playle and Wood, 1989; Playle *et al.* 1990). Compared with the gills, however, water is not continually renewed over the surface of the embryo. In fact, water surrounding the embryo may be relatively stagnant, as trout embryos may be buried in several inches of gravel (Orcutt *et al.* 1961).

In adult trout, the pH of the unstirred layer of the gills is determined by the rate of CO_2 and H^+ excretion (which result in a decrease in water pH) and NH_3 excretion (which results in an increase in water pH; Lin and Randall, 1990, 1991). Since the chorion is permeable to these molecules and oxidation of proteins is the main source of energy in embryos, we assume that the rates of CO_2, H^+ and NH_3 excretion together with the water buffering capacity determine the final pH of the unstirred layer next to the chorion in embryos.

We propose that the acid unstirred layer next to the chorion facilitates NH_3 excretion by maintaining the P_{NH_3} gradient, as in adult rainbow trout gills (Wright *et al.* 1989). As NH_3 is excreted across the chorion into the USL, NH_3 combines with H^+ to form NH_4^+. Total ammonia levels were higher in the unstirred layer than in the bulk water; therefore, only by reducing the pH of the USL can P_{NH_3} in the USL be minimized. It should be noted that calculated P_{NH_3} values in the PVF and USL were essentially the same under control conditions (Figs 1D, 2D); however, only a very small gradient would be necessary to maintain NH_3 diffusion.

The addition of borax buffer effectively eliminated the acid

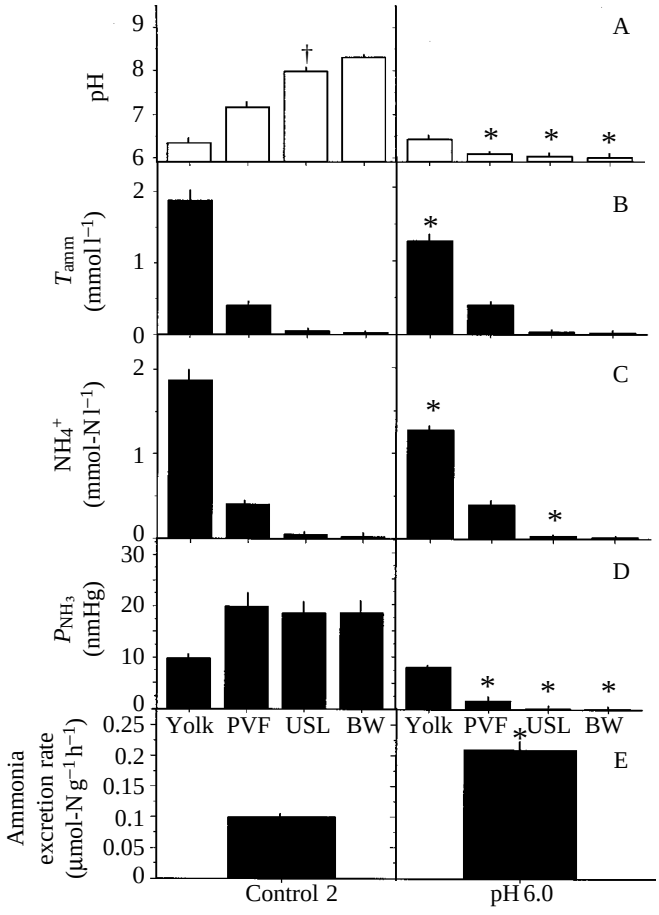

Fig. 2. A comparison between rainbow trout embryos exposed to control water 2 (see Materials and methods) at pH 8.3 or pH 6.0. (A) pH; (B) total ammonia-N concentration (T_{amm}); (C) [NH_4^+]; (D) partial pressure of NH_3 (P_{NH_3}); (E) ammonia excretion rates. See Fig. 1 for details.

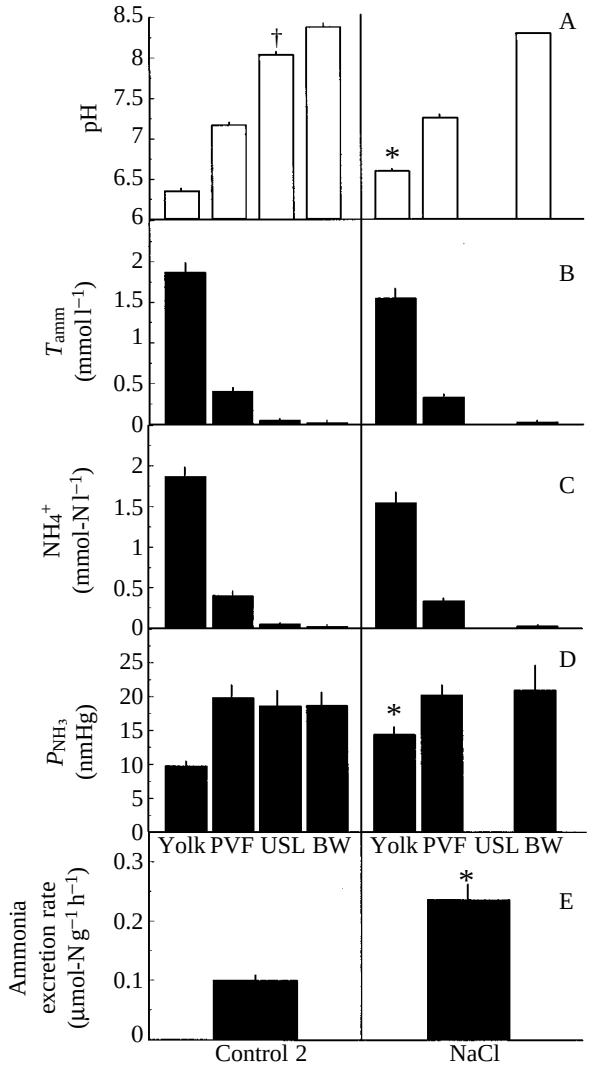

Fig. 3. A comparison between rainbow trout embryos exposed to control water 2 (see Materials and methods) of pH 8.3 or elevated external NaCl levels (1.6 mmol l^{-1}, pH 8.3). (A) pH; (B) total ammonia-N concentration (T_{amm}); (C) [NH_4^+]; (D) partial pressure of NH_3 (P_{NH_3}); (E) ammonia excretion rates. See Fig. 1 for details. For the NaCl experiment, pH and ammonia values were not measured in the USL. In addition, BW [NH_4^+] and P_{NH_3} were calculated from measured total ammonia concentrations (see Material and methods for further details).

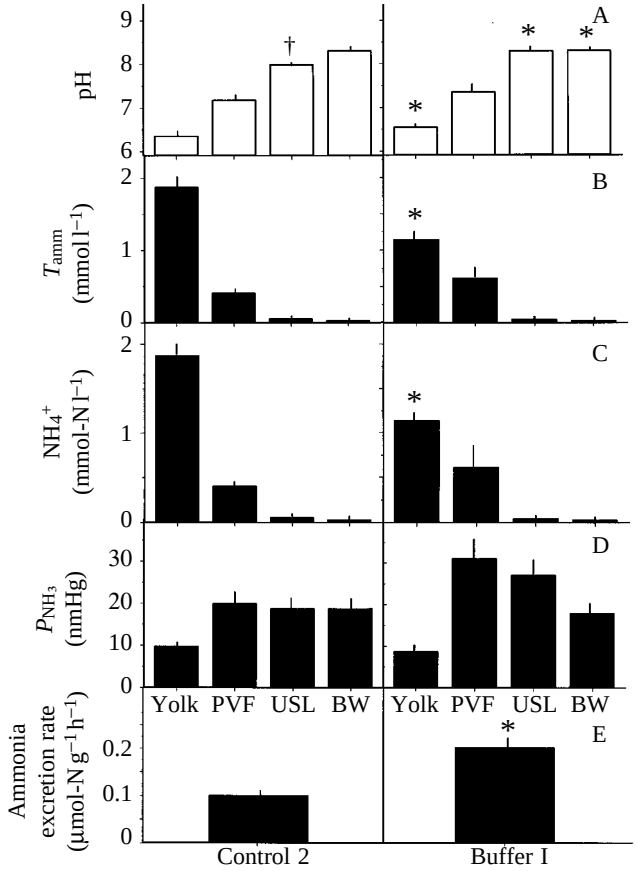

Fig. 4. A comparison between rainbow trout embryos exposed to control water 2 (see Materials and methods) at pH 8.3 or buffered water I (2.5 mmol l^{-1} $Na_2B_4O_7$, pH 8.3). (A) pH; (B) total ammonia-N concentration (T_{amm}); (C) [NH_4^+]; (D) partial pressure of NH_3 (P_{NH_3}); (E) ammonia excretion rates. See Fig. 1 for details.

USL (Fig. 4A), but we did not observe the expected decrease in ammonia excretion rate, possibly because of the fivefold elevation in external Na^+ levels (see below). The addition of a non-Na^+-based buffer, Hepes, resulted in the expected decrease in ammonia excretion rates (Fig. 5B), providing evidence for the influence of the acid USL on NH_3 diffusion.

In the adult rainbow trout, the presence of the enzyme carbonic anhydrase (CA) on the external surface of the gill catalyses the CO_2 hydration reaction as CO_2 is excreted across the gill epithelium into the environment (Wright *et al.* 1986). Thus, protons from the CO_2 hydration reaction are readily available for the NH_3 protonation reaction, thereby establishing a linkage between CO_2 and NH_3 excretion (Wright *et al.* 1989). It is not known whether CA is present on the external surface of the embryo; however, this seems unlikely since the chorion is an acellular layer. The uncatalysed CO_2 reaction is relatively slow, with a half-time of the order of minutes (Kern, 1960) at typical fish water pH and temperatures. Regardless of the presence or absence of CA, the USL is acidic relative to the BW, and the rate of ammonia excretion appears to be dependent on the maintenance of an acidic USL in trout embryos (see above).

Partitioning of ammonia between the yolk, PVF and USL

If NH_4^+ transfer across the chorion is negligible, and ammonia distribution is solely dependent on P_{NH_3} gradients, then one would expect the PVF-to-USL ammonia distribution to follow the H^+ distribution, as for other weak acids and bases with impermeant ion forms. Indeed, the distribution of ammonia between the USL and PVF is in agreement with the H^+ distribution because predicted USL T_{amm} was not significantly different from measured USL T_{amm} in both

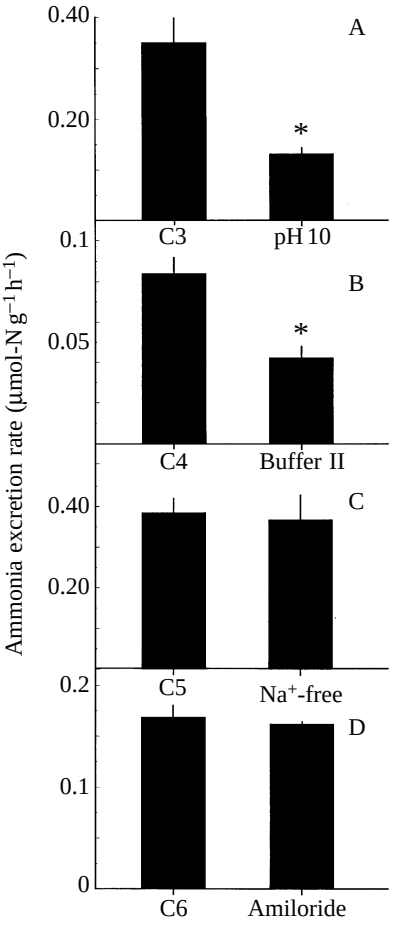

Fig. 5. Ammonia excretion rates in embryos exposed to (A) control 3 (C3) water (pH 8.3) or alkaline water (pH 10); (B) control 4 (C4) (pH 7.8) or buffered water II (2.5 mmol l^{-1} Hepes) at pH 7.8; (C) control 5 (C5) (pH 8.3) or 0.0 mmol l^{-1} NaCl (pH 8.3); (D) control 6 (C6) (pH 8.1) or 0.1 mmol l^{-1} amiloride (pH 7.9). Asterisks indicate that the experimental values were significantly different from the control measurements ($P \leq 0.05$). Values are means + S.E.M., N=4. See Materials and methods for further details.

control 1 and 2 groups (Table 1). In addition, the calculated $E_{NH_4^+}$ between the USL and PVF was substantially more negative than the measured E_M (Table 2), indicating that the NH_4^+ concentration was higher than that expected on the basis of the Nernst equilibrium across the chorion. Hence, it is probable that NH_3 is the dominant species of ammonia moving across the embryo under control conditions, whereas NH_4^+ is relatively impermeable.

Several researchers have reported that isolated chorions or intact embryos of freshwater teleosts are ion-selective (Eddy and Talbot, 1985; Peterson and Martin-Robichaud, 1987; Shephard and McWilliams, 1989). Both the chorion and the PVF contain negatively charged macromolecules (Peterson and Martin-Robichaud, 1986), and therefore the embryo can be compared with an 'ion exchanger' that has a distinct influence on electrolyte diffusion (Rudy and Potts, 1969; Peterson and Martin-Robichaud, 1986; Shephard and McWilliams, 1989). Moreover, Shephard and McWilliams (1989) reported that K^+ and Na^+ activities were not in equilibrium across the chorion of *Salmo salar* embryos. NH_4^+ has the same charge and hydrated ionic radius as K^+ and should mimic K^+ (Knepper *et al.* 1989). Hence, various published studies lend support to our conclusion that NH_4^+ is relatively impermeable across the chorion and that this may be due to properties of the chorion, the PVF or both.

If the yolk-sac membrane is relatively permeable to NH_4^+, then one would expect ammonia to be distributed between the yolk and the PVF according to the electrical potential and not the H^+ distribution. This was partly the case. The calculated $E_{NH_4^+}$ was approximately equal to the measured E_M between the PVF and yolk (Table 2), supporting the conclusion that NH_4^+ was in equilibrium across the membrane.

The pH gradient data were not as clear. In the control 2 experiment, the predicted yolk T_{amm} was significantly higher than the measured yolk T_{amm} (Table 1), indicating that ammonia was not distributed according to the H^+ distribution. The results discussed so far indicate that the yolk membrane is relatively permeable to NH_4^+. In the control 1 experiment, however, the predicted yolk T_{amm} was not significantly different from the measured T_{amm}, indicating that ammonia was distributed across the yolk membrane according to the H^+ distribution (Table 1). The discrepancy between the control 1 and 2 data may not be so surprising if one considers that the differences between measured and predicted values in the two control experiments were not that great compared with previous studies in fish tissues (Wright *et al.* 1988*a*). In rainbow trout muscle and lemon sole muscle, heart and brain the ratio of measured:predicted T_{amm} was between 7 and 18 (Wright and Wood, 1988; Wright *et al.* 1988*a*) compared with only 0.45 in the present study.

Mechanisms of ammonia excretion

Our findings support the hypothesis that ammonia is excreted, in part, by NH_3 diffusion down the P_{NH_3} gradient (Fig. 6). In most cases, changes in the P_{NH_3} gradients, but not the NH_4^+ gradients, were positively correlated with changes in ammonia excretion rate. Ammonia excretion was completely inhibited by high external ammonia levels, although the total external ammonia concentration was lower than that in either the PVF or the yolk. The P_{NH_3} gradient in this case, however, was reversed; that is, from the BW to the PVF. In contrast, the NH_4^+ concentration gradient from the yolk to the BW was positive, which indicates that NH_4^+ diffusion was not the main mechanism of ammonia excretion under these conditions. [It should be noted that the data do not allow us to differentiate between excretion directly from the embryo to the PVF (and to the external medium) or from the embryo to the yolk and from the yolk to the PVF (and to the external medium) (Fig. 6). Amino acid deamination probably occurs primarily in the developing embryonic liver, as in adult teleosts (Forster and Goldstein, 1969). The yolk syncytium surrounding the yolk sac is responsible for the release of yolk metabolites (Heming and Buddington, 1988). The yolk itself

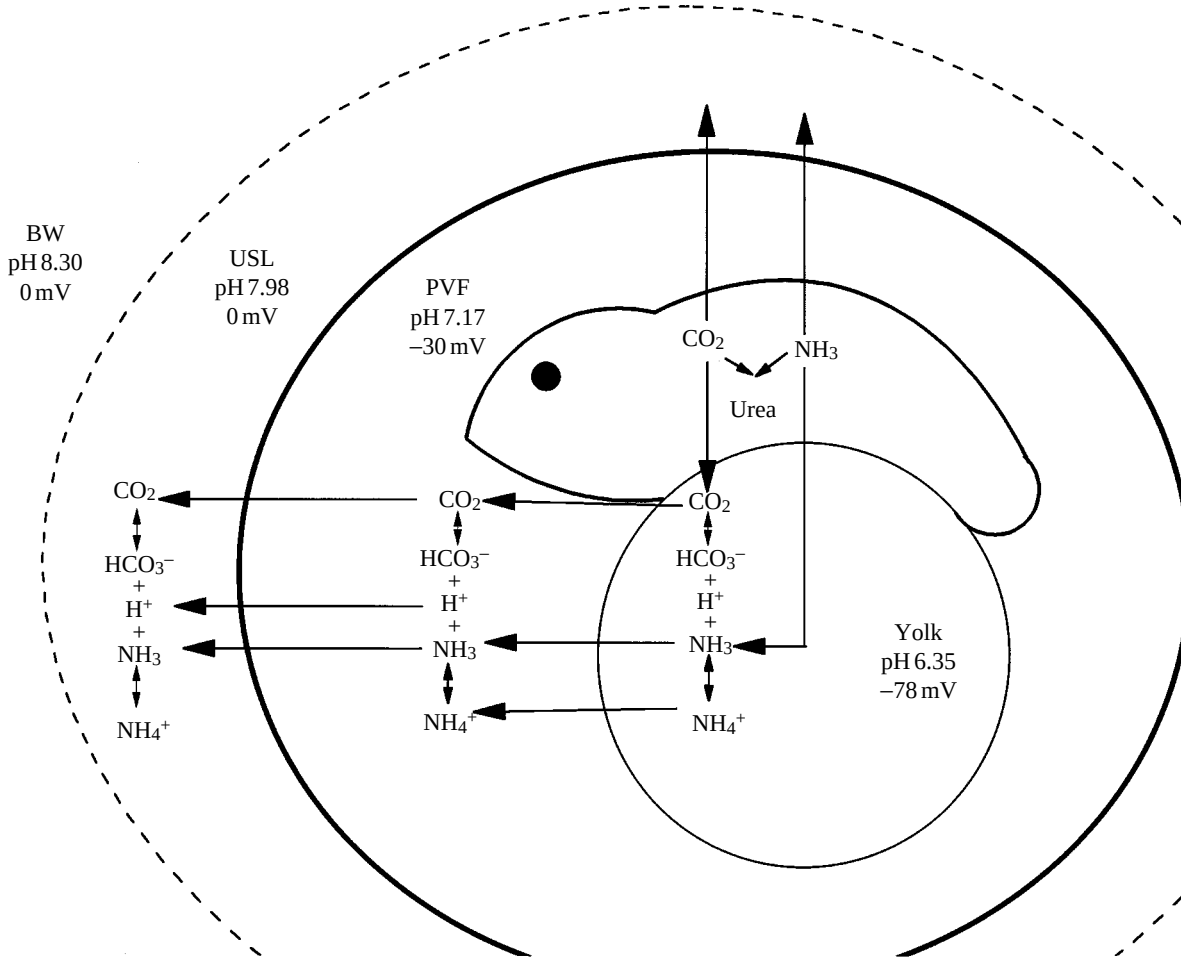

Fig. 6. A model proposed for ammonia excretion from the embryo to the environment under control conditions. BW, bulk water; USL, unstirred layer; PVF, perivitelline fluid. The figure is not drawn to scale. Excretion pathways may be directly from the yolk to the PVF to the USL and/or from the embryo to the PVF to the USL. For simplicity, excretion pathways are drawn only from the yolk. Although there are no published data on the permeability of the chorion to HCO_3^-, it is likely that the majority of carbon dioxide exits the embryo to the external medium as CO_2, as in adult fish (Perry, 1986). In addition, Shephard and McWilliams (1989) reported that Cl^- is less permeable across the chorion than is H^+ or K^+. Although direct measurements of HCO_3^- permeability are beyond the scope of this paper, our model assumes that HCO_3^- permeability is similar to that of Cl^- and much less than that of CO_2. Clearly, these assumptions need to be validated with experimental measurements. Our data indicate that the yolk membrane and/or the embryo are relatively impermeable to H^+ compared with the chorion. In contrast, the yolk membrane and/or embryo are relatively permeable to NH_4^+ compared with the chorion.

also possesses enzymes (Hamor and Garside, 1977) that may be involved in the breakdown of the yolk. Thus, the specific site(s) of ammonia production has yet to be defined in fish embryos.]

Acidification of the external water increased the P_{NH_3} gradient from the yolk to the PVF and from the PVF to the unstirred water layer, while the NH_4^+ gradient was unchanged or even decreased. The 100 % increase in ammonia excretion rate, therefore, indicates that a significant proportion of ammonia was excreted as NH_3 under these conditions.

Exposure of the embryos to severely alkaline water (pH 10) also decreased ammonia excretion rate. This reduction presumably reflects a change in the P_{NH_3} gradient between BW and PVF. High external pH is also known to inhibit or greatly reduce ammonia excretion in hatched embryos and alevin (P. A. Wright and M. Land, in preparation) and adult rainbow trout (Wright and Wood, 1985; Wilkie and Wood, 1991; Yesaki and Iwama, 1992). Furthermore, elimination of the acid USL by addition of a strong buffer (Hepes) to the external water presumably reduced the P_{NH_3} gradient from PVF to USL, resulting in 50 % inhibition of ammonia excretion. Taken together, these data indicate that NH_3 plays a dominant role in ammonia excretion in embryos of rainbow trout.

It is interesting to note that our calculations of P_{NH_3} values for yolk and PVF were very similar to those calculated by Cameron and Heisler (1983) and by Wright and Wood (1985) for adult rainbow trout plasma. Under control conditions, adult rainbow trout have a P_{NH_3} diffusion gradient from blood to water of 38–57 nmHg (Wright and Wood, 1985). In the present study, gradients between different compartments

(yolk, PVF, USL and BW) in and around the embryo were 1–45 nmHg. The diffusion pathway across the gills of adult trout may be more complex compared with the diffusion pathway across the yolk membrane and the chorion in embryonic trout. Both the adult and embryo, however, are exposed to the same external environment and must eliminate ammonia to prevent toxic levels from accumulating in the tissues. Hence, it may not be too surprising that the gradient for NH_3 diffusion is of the same order of magnitude in the embryo and adult.

A $Na^+/H^+(NH_4^+)$ exchange has been proposed for juvenile steelhead trout (Bradly and Rourke, 1985) and adult rainbow trout (Wright and Wood, 1985; McDonald and Prior, 1988). Wilson *et al.* (1994) and Avella and Bornancin (1989), however, have rejected the hypothesis that NH_4^+ excretion is directly linked to Na^+ uptake at the gills of rainbow trout. If a Na^+-dependent NH_4^+ transport system [e.g. a $Na^+/H^+(NH_4^+)$ exchanger] were involved in ammonia excretion from the embryo to the external water (i.e. on the embryo and/or yolk-sac membrane), an increase or decrease in external [Na^+] should result in a corresponding change in ammonia excretion rate. In addition, exposure to amiloride, an inhibitor of the $Na^+/H^+(NH_4^+)$ transporter and Na^+ channels, should significantly reduce ammonia excretion rate. Exposure of embryos to elevated NaCl levels resulted in a twofold increase in ammonia excretion rate, providing evidence for some involvement of Na^+ in ammonia excretion. However, ammonia excretion was not significantly affected by the removal of Na^+ from the external water or by amiloride treatment. Hence, it is unlikely that a $Na^+/H^+(NH_4^+)$ transporter is directly involved in ammonia excretion. We do not know, however, whether the effect of Na^+ on ammonia excretion is related to changes in pH or E_M, or has general effects on embryo health.

In summary, we propose that ammonia is primarily excreted by NH_3 diffusion down the P_{NH_3} gradient (Fig. 6). Some ammonia may be converted to urea *via* the ornithine–urea cycle or uricolytic pathways (Dépêche *et al.* 1979; Wright *et al.* 1995). In a companion study, we have found that urea levels in the yolk are considerably higher than in the PVF and that urea-N excretion accounts for between 20 and 50 % of total nitrogen excretion (ammonia-N + urea-N) in trout embryos (E. Rahaman-Noronha, M. J. O'Donnell, K. Moore and P. A. Wright, in preparation). Thus, nitrogen excretion in rainbow trout embryos is not simply accomplished by ammonia elimination, as was previously thought (Smith, 1947; Rice and Stokes, 1974; Wright *et al.* 1995), but also involves the more chemically complex nitrogen end-product urea.

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