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Occludin and hydromineral balance in *Xenopus laevis*

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SUMMARY

To investigate the response of the tight junction (TJ) protein occludin to environmental change in an anuran amphibian, we examined occludin tissue distribution, immunolocalization and alterations in mRNA expression in African clawed frogs (*Xenopus laevis*) acclimated to brackish water (BW) conditions (from freshwater to 2‰, 5‰ or 10‰ salt water). Occludin mRNA is widely expressed in *Xenopus* and is abundant in tissues involved in regulating salt and water balance, such as the gastrointestinal (GI) tract, kidney and urinary bladder. Immunohistochemical analyses revealed strong occludin immunolabelling in the apicolateral region of epithelia lining the GI tract and mRNA expression increased along the longitudinal axis of the gut. In kidney tissue, occludin was differentially expressed on the luminal side of the nephron tubule, appearing in the distal tubules and collecting ducts only. In response to BW acclimation, *Xenopus* exhibited a significant loss of tissue water as well as salinity-dependent elevations in serum osmolality as a result of increased urea levels followed by elevated serum Na^+ and Cl^- levels. Tissue-specific alterations in the ionomotive enzyme Na^+,K^+-ATPase were also observed in *Xenopus* in response to BW acclimation. Most notably, Na^+,K^+-ATPase activity in the rectum increased in response to elevated environmental salt concentrations while renal activity decreased. Furthermore, acclimation to BW caused tissue-specific and salinity-dependent alterations in occludin mRNA expression within select *Xenopus* osmoregulatory organs. Taken together, these studies suggest that alterations in occludin, in conjunction with active transport processes, may contribute to amphibian hydromineral homeostasis during environmental change.

Key words: tight junction, occludin, hydromineral balance, Na^+,K^+-ATPase, paracellular permeability, osmoregulation, amphibian.

INTRODUCTION

The osmoregulatory capabilities of aquatic vertebrates are defined by the barrier and transport properties of epithelial tissues such as the skin, gut, kidney, gill and urinary bladder. These epithelia either separate and modify body fluids internally or directly contact the environment and regulate water and solute exchange between internal and external compartments. Anuran amphibians have served as classic model systems for investigating the transport properties of epithelia, particularly the active and passive transfer of solutes across the transcellular and paracellular pathways, respectively. However, while mechanisms of transport across the transcellular pathway have been well characterized (e.g. Leaf, 1982; Nedergaard et al., 1999; Dantzler, 2003), less is know about the tight junction (TJ) complex and the molecular components that control the paracellular route.

As the apical-most component of the cell–cell junctional complex, the TJ forms a semi-permeable paracellular barrier that limits the movement of water and solutes between the apical and basolateral compartments of an epithelium (Cereijido and Anderson, 2001). TJ proteins, which exist as either integral transmembrane proteins or associated cytosolic proteins, organize into fibrils at sites of cell–cell contact and, by freeze-fracture microscopy, appear as networks of strands encircling the apical domains of epithelial cells (Claude and Goodenough, 1973; González-Mariscal et al., 2003). Electrophysiological measurements across amphibian epithelia in conjunction with freeze-fracture electron microscopy highlighted the elementary relationship between junctional morphology and paracellular permeability. While ‘leaky’ epithelia (e.g. proximal tubules of *Necturus* kidney) possess simple TJs composed of one to two strands, ‘tighter’ epithelia (e.g. frog urinary bladder) exhibit complex networks of several TJ strands (Claude and Goodenough, 1973; Humbert et al., 1976). Occludin, a transmembrane protein of the TJ complex, localizes exclusively to TJ strands at sites of cell–cell contact (Furuse et al., 1993). Homotypic associations between occludin within apposing TJ strands of adjacent cells are understood to play a role in the regulation of permeability or ‘tightness’ of the paracellular barrier (González-Mariscal et al., 2003; Feldman et al., 2005). For example, administration of synthetic peptides corresponding to the extracellular domains of occludin led to decreased transepithelial resistance (TER) and increased paracellular flux across *Xenopus* A6 epithelia (Wong and Gumbiner, 1997; Lacaz-Vieira et al., 1999). Additionally, over-expression of occludin in Madin–Darby canine kidney cells significantly increased TER and correspondingly increased the mean number of TJ strands within cells (Balda et al., 1996; McCarthy et al., 1996). Occludin expression, at both the mRNA and protein level, has become a reliable indicator of paracellular permeability, and thus epithelial ‘tightness’, as an extensive number of studies have demonstrated a well-defined correlation between occludin expression, TER and paracellular flux in a wide variety of tissues both *in vivo* and *in vitro* (e.g. Antonetti et al., 2002; Demaude et al., 2006; Al-Sadi and Ma, 2007; Colgan et al., 2007).

The freshwater (FW) African clawed frog, *Xenopus laevis*, is remarkably tolerant of elevated salinity and water deprivation (Munsey, 1972; Jørgensen, 1997). Its ability to acclimate from FW to saline conditions reflects a successful interplay of osmoregulatory strategies that shift between eliminating excess water and combating obligatory ion loss in a FW environment to conserving water and limiting excessive ionic uptake or retention while under saline conditions. In order to maintain salt and water balance when

environmental perturbation occurs, changes in transcellular transport processes must take place. Furthermore, several lines of evidence (mainly electrophysiological analyses) have suggested that water and salt exchange across the paracellular pathway can significantly contribute to processes of amphibian hydromineral homeostasis (Leaf, 1982; Nedergaard et al., 1999; Dantzler, 2003). However, to date, TJ protein studies in amphibians have focused largely on TJ assembly during *Xenopus* embryogenesis (Cardellini et al., 1996; Cordenonsi et al., 1997; Fesenko et al., 2000; Fujita et al., 2002) and, to the best of our knowledge, no studies have comprehensively examined the response of amphibian TJ proteins to environmental change. Therefore, in the present study, our aim was to establish a potential role for occludin in the regulation of hydromineral balance in amphibia. To accomplish this, we characterized the distribution and localization of occludin within non-embryonic *Xenopus* tissues, and examined alterations in hydromineral endpoints, ionomotive enzyme activity and occludin mRNA expression in response to environmental change by means of brackish water (BW) acclimation. We hypothesized that occludin mRNA expression would alter in response to elevated environmental salt content in a salinity-dependent and tissue-specific manner.

MATERIALS AND METHODS

Animals

African clawed frogs (*Xenopus laevis*, Daudin; 5.62±0.3 g) were obtained from a local supplier and held for at least 4 weeks in 60 l glass aquaria containing dechlorinated FW (approximate composition in mmol l^{-1}: Na^+, 0.59; Cl^-, 0.92; Ca^{2+}, 0.76; and K^+, 0.043) at room temperature (~21°C). Animals were held at a constant photoperiod cycle of 12 h light/12 h dark and were fed *ad libitum* once daily with BioPure® blood worms (Hikari Sales, Hayward, CA, USA). *Xenopus* were then randomly separated into four groups and held in 8.5 l opaque polyethylene tanks containing dechlorinated FW at a density of six to eight frogs per tank. Following a 1 week settling period, three of the four groups of frogs were gradually acclimated to BW of varying salinity (2‰, 5‰ or 10‰) by addition of Instant Ocean™ synthetic sea salt (Aquarium Systems, Sarrebourg, France) at a rate of 1–2‰ per day. Once the desired salinity of each group was achieved, the frogs were allowed to acclimate to their new environment for 2 weeks. The salinity of each experimental group was confirmed and monitored daily with a hand-held refractometer (SR-6 VitalSine Refractometer, Rhinelander, WI, USA). During the course of the BW acclimation experiments, animals were fed as described above; however, no food was provided 24 h prior to sampling.

Blood and tissue collection

For all experiments, *Xenopus* were net captured and anaesthetized using 1 g l^{-1} tricaine methanesulphonate (MS-222; Syndel Laboratories, Qualicum Beach, BC, Canada) prepared in water of appropriate salinity. Tissues collected for expression profile analysis were carefully dissected from stock FW animals, quick frozen in liquid nitrogen and stored at –80°C until further analysis. The following tissues were collected: brain, eye, heart, lung, stomach, anterior intestine, posterior intestine, rectum, liver, gallbladder, spleen, kidney, urinary bladder, dorsal skin, ventral skin, muscle, adipose tissue and blood. Blood tissue used for RNA extraction consisted of packed blood cells separated from serum following centrifugation in a micro-haematocrit tube (see below). The anterior and posterior intestinal regions are defined as the anterior and posterior areas of the small intestine between the pylorus of the stomach and the sphincter leading to the large intestine (i.e. rectum). This portion of the gastrointestinal (GI) tract was measured lengthwise and divided equally into two portions. For histological analysis, regions of the GI tract (as previously described) and kidney were collected from FW *Xenopus* and immediately fixed in Bouin's solution for 3–4 h followed by storage in 70% ethanol at 4°C until further processing.

For experiments in which *Xenopus* had been acclimated to BW conditions, anaesthetized frogs were quickly rinsed with distilled water and blotted dry, and blood was sampled into micro-haematocrit capillary tubes (Fisher Scientific, Pittsburgh, PA, USA) following spinal transection. Blood was allowed to clot at room temperature for 30 min and was centrifuged for 5 min at 9500 ***g*** using a Haematokrit 20 centrifuge (Hettich Zentrifugen, Tuttlingen, Germany). Serum was separated from the pellet of packed blood cells and stored at –30°C until further use. To examine changes in occludin mRNA expression in response to elevated environmental salinity, regions of the GI tract, kidney, urinary bladder and dorsal and ventral skin were collected from FW and 2‰, 5‰ and 10‰ BW-acclimated *Xenopus* for RNA extraction. Additionally, regions of the GI tract, kidney and urinary bladder were collected for Na^+,K^+-ATPase activity analysis. All tissue samples collected for RNA extraction and Na^+,K^+-ATPase activity analysis were quick frozen in liquid nitrogen and stored at –80°C until further use. A standardized region of *Xenopus* leg muscle (sartorius) was also removed for analysis of muscle moisture content. All experiments were carried out in accordance with the principles published in the Canadian Council on Animal Care's guide to the care and use of experimental animals.

Occludin expression profile

Reverse transcriptase PCR (RT-PCR) was used to examine occludin mRNA distribution and expression in *Xenopus* tissues. Total RNA was extracted from *Xenopus* tissues using TRIzol® Reagent (Invitrogen Canada, Burlington, ON, Canada) as per the manufacturer's instructions following homogenization using a PRO250 homogenizer (Pro Scientific Inc., Oxford, CT, USA). All RNA samples were treated with DNase I (Amplification Grade; Invitrogen Canada) prior to cDNA synthesis. SuperScript™ III Reverse Transcriptase and Oligo$(dT)_{12–18}$ primers (Invitrogen Canada) were used to generate first-strand cDNA from DNase I-treated RNA samples. Occludin primers (forward 5′-TTGCGT-GTGTGGCTTCAAC-3′ and reverse 5′-CTCCTACGGTATAA-ACAATGGTCC-3′, predicted amplicon size 351 bp) were designed using a previously published *Xenopus* occludin coding sequence as a template (Cordenonsi et al., 1999) (GenBank accession no. NM 001088474). For use as an internal control, β-actin primers (forward 5′-GTGACCTGACAGACTACCTC-3′ and reverse 5′-GTACCACCAGACAGAACAG-3′, predicted amplicon size 361 bp) were designed based on GenBank accession no. NM 001088953.

RT-PCR amplification of occludin (and of β-actin as an internal control) was performed (using 0.2 mmol l^{-1} dNTPs, 0.2 μmol l^{-1} forward and reverse primers, 1×Taq DNA polymerase buffer, 1.5 mmol l^{-1} $MgCl_2$ and 1 U Taq DNA polymerase; Invitrogen Canada) under the following reaction conditions: 1 cycle of denaturation (95°C, 4 min), 40 cycles of denaturation (95°C, 30 s), annealing (58°C for occludin or 51°C for β-actin, 30 s) and extension (72°C, 30 s), final single extension cycle (72°C, 5 min). Final PCR products were resolved electrophoretically in 1% agarose gels for approximately 90 min at 100 V, and stained with ethidium bromide. Images used for expression profiles were captured using a MultiImage™ Light Cabinet (AlphaImager® HP model; Alpha Innotech Corp., San Leandro, CT, USA).

Histology and immunohistochemistry

Fixed tissues stored in 70% ethanol were dehydrated and embedded in Paraplast Plus tissue embedding medium (Oxford Worldwide, LLC, Memphis, TN, USA). To examine the dorsoventral organization of the *Xenopus* kidney, longitudinal sections (6 μm thick) were stained with haematoxylin and eosin. Occludin and Na^+,K^+-ATPase immunolocalization in *Xenopus* tissue was examined using methods previously outlined (Chasiotis and Kelly, 2008). Briefly, deparaffinized and rehydrated sections (4 μm thick) were subjected to heat-induced epitope retrieval, quenched with 3% H_2O_2, washed and then incubated overnight at room temperature with rabbit polyclonal anti-occludin antibody [1:100 dilution in antibody dilution buffer (ADB); Zymed Laboratories, South San Francisco, CA, USA] and mouse monoclonal anti-Na^+,K^+-ATPase α-subunit antibody (α5; 1:10 in ADB; Developmental Studies Hybridoma Bank, Iowa City, IA, USA). After washing, sections were incubated with TRITC-labelled goat anti-rabbit antibody (1:500 in ADB; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and FITC-labelled goat anti-mouse antibody (1:500 in ADB; Jackson ImmunoResearch Laboratories) for 1 h at 37°C. Sections were washed once more, allowed to air dry for 1 h and then mounted with Molecular Probes ProLong Antifade (Invitrogen Canada) containing 5 μg ml^{-1} DAPI (Sigma-Aldrich Canada, Oakville, ON, Canada). Fluorescence images were captured using a Reichert Polyvar microscope (Reichert Microscope Services, Depew, NY, USA) and an Olympus DP70 camera (Olympus Canada, Markham, ON, Canada). Adobe Photoshop CS2 software was used for contrast and brightness adjustment of entire images (Adobe Systems Canada, Toronto, ON, Canada). Control sections were also prepared for each tissue examined by omitting primary antibodies from overnight incubation.

Serum analysis, Na^+,K^+-ATPase enzyme activity and muscle moisture content

Serum osmolality was determined using a Model 5500 Vapor Pressure Osmometer (Wescor, Logan, UT, USA). Serum Na^+ levels were measured using an atomic absorption spectrometer (AAnalyst 200 spectrometer, PerkinElmer Life and Analytical Sciences, Woodbridge, ON, Canada) and serum Cl^- and urea concentrations were determined using colorimetric assays previously described (Zall et al., 1956; Rahmatullah and Boyde, 1980). *Xenopus* tissues collected for Na^+,K^+-ATPase activity analysis were homogenized on ice in a pre-chilled SEI (150 mmol l^{-1} sucrose, 10 mmol l^{-1} EDTA, 50 mmol l^{-1} imidazole, pH 7.3):SEID (0.5 g sodium deoxycholate/100 ml SEI) buffer mixture (4:1 mixture of SEI:SEID) using a PRO250 homogenizer. Homogenates were centrifuged at 3200 ***g*** for 10 min at 4°C and supernatants were collected, quick frozen in liquid nitrogen and stored at –80°C until enzyme analysis. Supernatants were thawed on ice and assayed for Na^+,K^+-ATPase activity using methods and according to conditions previously outlined (Giunta et al., 1984; McCormick, 1993). For analysis of muscle moisture content, *Xenopus* leg muscle was dried to a constant weight at 60°C for 1 week. Moisture content was subsequently determined gravimetrically.

Quantitative real-time PCR analysis (qRT-PCR)

qRT-PCR analysis was carried out using a Chromo4^{TM} Detection System (CFB-3240, Bio-Rad Laboratories, Mississauga, ON, Canada) and SYBR Green I Supermix (Bio-Rad Laboratories). qRT-PCR amplifications of occludin (and β-actin as an internal control), using the primers and cDNA described above, were performed under the following conditions: 1 cycle of denaturation (95°C, 4 min) followed by 40 cycles of denaturation (95°C, 30 s), annealing (58°C for occludin or 51°C for β-actin, 30 s) and extension (72°C, 30 s). To ensure that no primer dimers or other non-specific products were synthesized during reactions, a melting curve analysis was carried out after each qRT-PCR run.

Statistical analysis

All data are expressed as mean values ± s.e.m. To examine for statistical significance between groups, a one-way analysis of variance (ANOVA) was used. If the ANOVA test indicated significance ($P \leq 0.05$), it was followed by a Student–Newman–Keuls multiple comparison test. All statistical analyses were conducted using Graphpad Instat Software Version 3.00 (GraphPad Software, San Diego, CA, USA).

RESULTS

Tissue distribution of *Xenopus* occludin mRNA

Occludin mRNA was found to be widely expressed in *Xenopus* tissues (Fig. 1). While moderate to strong expression was found in heart, lung, stomach, anterior and posterior intestine, liver, gallbladder, kidney, dorsal and ventral skin, and adipose tissues, very strong expression was detected in the rectum and urinary bladder. Weak levels of expression were found in brain, eye and spleen. No occludin mRNA expression was detected in muscle and blood tissue. β-Actin mRNA, used as an internal control, was highly expressed in all tissues examined, except the eye and blood (Fig. 1).

Occludin immunolocalization in *Xenopus* GI tract and kidney

In all regions of the GI tract, occludin immunolocalized to apicolateral regions of epithelia lining the lumen and exhibited a honeycomb-like pattern when cells were sectioned transversely (Fig. 2A–D). This contrasted with Na^+,K^+-ATPase immunoreactivity, which was detected along the basolateral membranes of enterocytes lining the mucosa of the anterior and posterior intestine as well as the rectum (Fig. 2B–D). Little to no Na^+,K^+-ATPase immunostaining was detected within stomach tissue (Fig. 2A). In the stomach, occludin immunostaining appeared to be restricted to apicolateral sites of cell–cell contact, while occludin immunofluorescence labelling in the small intestine and rectum appeared less punctate and more uniformly distributed along entire apical surfaces of enterocytes (Fig. 2A–D). Although no discernable occludin gradient along the villus to crypt axis was observed within the small intestine and rectum, enhanced occludin immunostaining was observed in the stomach between cells at the base of the gastric pits (Fig. 2A, arrow). There was also no discernable occludin gradient along the longitudinal axis of the GI tract from the anterior intestine to the rectum (Fig. 2B–D). Control stomach, small intestine and rectum sections, probed with secondary antibody only, showed no TRITC or FITC fluorescence (Fig. 2E,F).

Based on the dorsoventral organization of the amphibian kidney and the differential expression of Na^+,K^+-ATPase along the nephron of *Xenopus*, discrete regions within the *Xenopus* nephron were easily identified. This allowed the localization and distribution of occludin along the nephron to be examined. Similar to other amphibians, the *Xenopus* kidney can be divided into two zones: the ventromedial zone and the dorsolateral zone (Stewart, 1927; Uchiyama and Yoshizawa, 2002). Separated by a medial band of glomeruli, the ventromedial zone contains mainly distal nephron segments while the dorsolateral zone contains all other nephron segments (i.e. proximal tubules and collecting ducts; Fig. 3A). Na^+,K^+-ATPase immunolocalization patterns along the nephron matched those previously described (Uchiyama and Yoshizawa, 2002). Briefly,

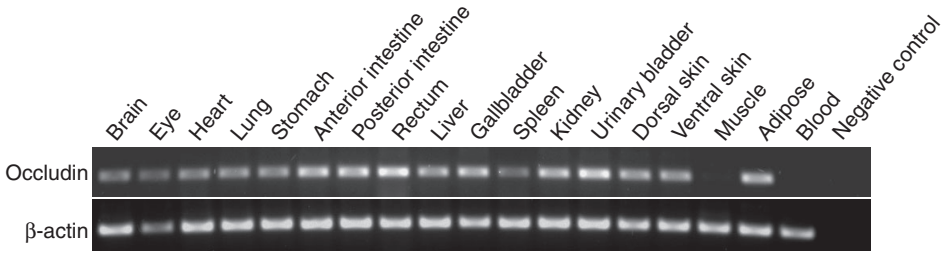

Fig. 1. Distribution of occludin mRNA in *Xenopus* tissues by RT-PCR analysis. *Xenopus* β-actin (bottom panel) was used as a loading control. A negative control for each gene was also run. Amplicon size for occludin and β-actin was 351 and 361 bp, respectively.

weak to undetectable Na^+,K^+-ATPase immunostaining was observed along the basal membrane of proximal tubule cells while the basolateral membranes of early distal tubule cells exhibited very strong Na^+,K^+-ATPase immunoreactivity (Fig. 3B–D). Na^+,K^+-ATPase also immunolocalized to the basolateral membranes of late distal tubule cells and collecting duct cells; however, staining intensity was moderate and immunofluorescence labelling was restricted to membranes of only one cell type (e.g. principal or intercalated cell), resulting in a periodic staining appearance (Fig. 3B,E,F). Dual-immunofluorescence labelling of occludin and Na^+,K^+-ATPase demonstrated differential apical localization of occludin within certain segments of the *Xenopus* nephron. While no occludin immunoreactivity could be detected in proximal segments of the nephron, strong occludin expression was detected in distal tubules (both early and late) and collecting ducts (Fig. 3C–F). No discernable differences in staining intensity were observed between distal and collecting segments; however, occludin immunofluorescence labelling in the collecting tubule appeared less punctate and more uniform (Fig. 3E,F). Control kidney sections, probed with secondary antibody only, showed no TRITC or FITC fluorescence (Fig. 3G).

Serum composition, muscle moisture content and Na^+,K^+-ATPase activity in response to BW acclimation

Acclimation of *Xenopus* to BW conditions resulted in salinity-dependent elevations in serum osmolality and urea levels (Fig. 4A,B). These changes were seen in frogs held in 5‰ and most notably 10‰ BW. Relative to the animals held in FW, serum Na^+ and Cl^- concentrations and muscle moisture content did not significantly alter in response to 2‰ and 5‰ BW acclimation (Fig. 4C–E). However, the 10‰ BW-acclimated group exhibited a significant increase in both serum Na^+ and Cl^- concentrations and a significant decrease in muscle moisture content compared with the FW group and all other BW treatment groups (Fig. 4C–E). Na^+,K^+-ATPase activity in *Xenopus* stomach significantly increased in response to 2‰ and 5‰ BW acclimation (Table 1). Stomach enzyme activity in 10‰ BW-acclimated frogs, however, did not significantly differ from the FW group (Table 1). Na^+,K^+-ATPase

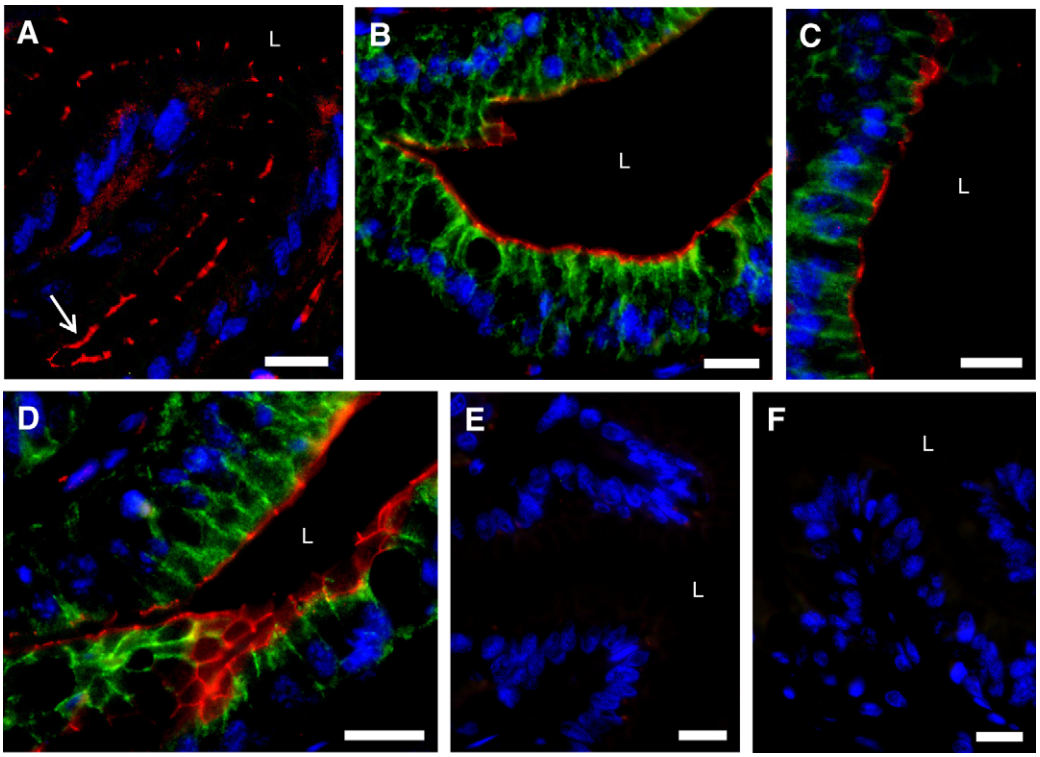

Fig. 2. Occludin and Na^+,K^+-ATPase immunolocalization in cross-sections of *Xenopus* (A) stomach, (B) anterior intestine, (C) posterior intestine and (D) rectum. Occludin (red) immunolocalized to apicolateral membranes of surface epithelial cells lining the stomach mucosa and enterocytes of the small intestine and rectum. Enhanced occludin immunostaining was also observed in the stomach between cells at the base of the gastric pits (arrow). Na^+,K^+-ATPase (green) was undetectable in the stomach but immunolocalized basolaterally in the small intestine and rectum. Control sections, probed with secondary antibody only, are shown in E and F for the stomach and rectum, respectively. L, lumen. Scale bars, 20 μm.

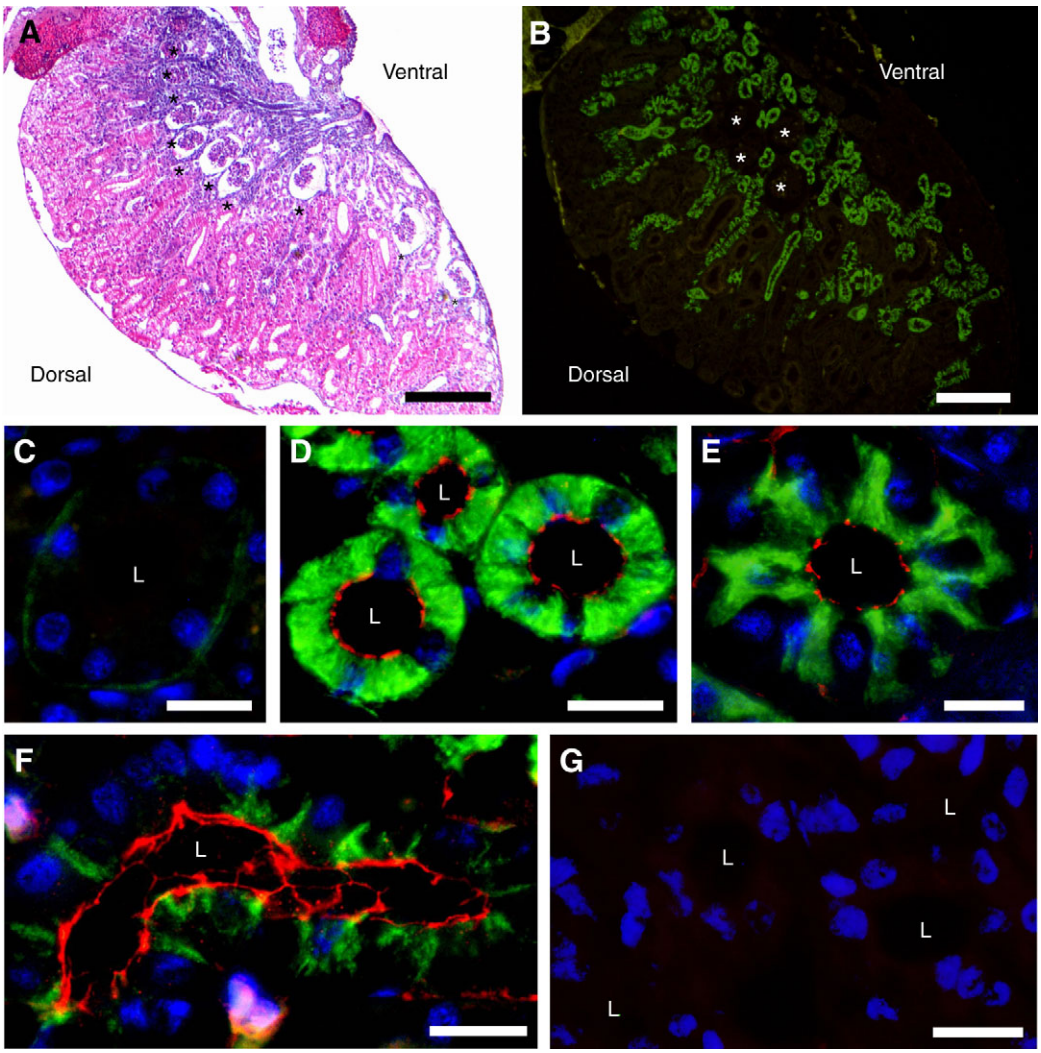

Fig. 3. (A) Longitudinal section of the *Xenopus* kidney stained with haematoxylin and eosin. A medial band of glomeruli (*) separates the ventromedial zone from the dorsolateral zone of the *Xenopus* kidney. Proximal tubules and collecting ducts are contained within the dorsolateral zone, while distal tubules are located within the ventromedial zone. (B) Na^+,K^+-ATPase immunostaining (green) within a longitudinal section of the *Xenopus* kidney. Within the dorsolateral zone, only collecting ducts show Na^+,K^+-ATPase immunoreactivity. Within the ventromedial zone, distal tubules (both early and late) show immunofluorescence labelling for Na^+,K^+-ATPase. (C–F) Dual-immunofluorescence labelling of occludin and Na^+,K^+-ATPase within cross-sections of the (C) proximal tubule, (D) early distal tubule, (E) late distal tubule and (F) collecting duct of the *Xenopus* nephron. Occludin (red) and Na^+,K^+-ATPase (green) were differentially expressed in the nephron. Occludin was undetectable in proximal tubules, but immunolocalized to apical membranes of renal epithelial cells of distal tubules (early and late) and the collecting duct. Na^+,K^+-ATPase weakly immunolocalized basally in proximal tubules and exhibited robust basolateral localization in the distal and collecting segments. A control section, probed with secondary antibody only, is shown in G. L, lumen. Scale bars for A and B, 300 μm; scale bars for C–G, 20 μm.

activity in the anterior intestine and posterior intestine did not significantly alter in response to elevated salinity, while enzyme activity in the rectum exhibited a stepwise increase, displaying an approximately 34% and 45% elevation in response to 5‰ and 10‰ BW acclimation, respectively, relative to FW animals (Table 1). While Na^+,K^+-ATPase activity in the kidney significantly decreased in response to 2‰, 5‰ and 10‰ BW acclimation, Na^+,K^+-ATPase activity in the urinary bladder of 10‰ BW-acclimated frogs did not significantly differ from the FW group (Table 1).

qRT-PCR analysis of occludin mRNA expression in *Xenopus* acclimated to BW

In FW frogs, occludin mRNA expression increased along the longitudinal axis of the GI tract (i.e. stomach < anterior and posterior intestine < rectum; Fig. 5A). When acclimated to BW conditions, significant tissue-specific and salinity-dependent alterations in occludin mRNA expression occurred. BW acclimation did not significantly alter stomach (data not shown) or posterior intestine occludin mRNA expression (Fig. 5C). However, acclimation to 10‰ BW significantly decreased anterior intestine occludin mRNA expression (Fig. 5B), and 5‰ and 10‰ BW conditions significantly increased rectal occludin mRNA expression (Fig. 5D). Occludin mRNA expression in the *Xenopus* kidney significantly increased in a salinity-dependent manner (Fig. 6A). In the urinary bladder, occludin mRNA expression appeared to exhibit a decline in response to BW conditions, relative to frogs held in FW; however, this reduction was only significant in the 10‰ BW-acclimated group (Fig. 6B). Occludin mRNA expression in the dorsal and ventral skin did not significantly change in response to BW acclimation (Fig. 7).

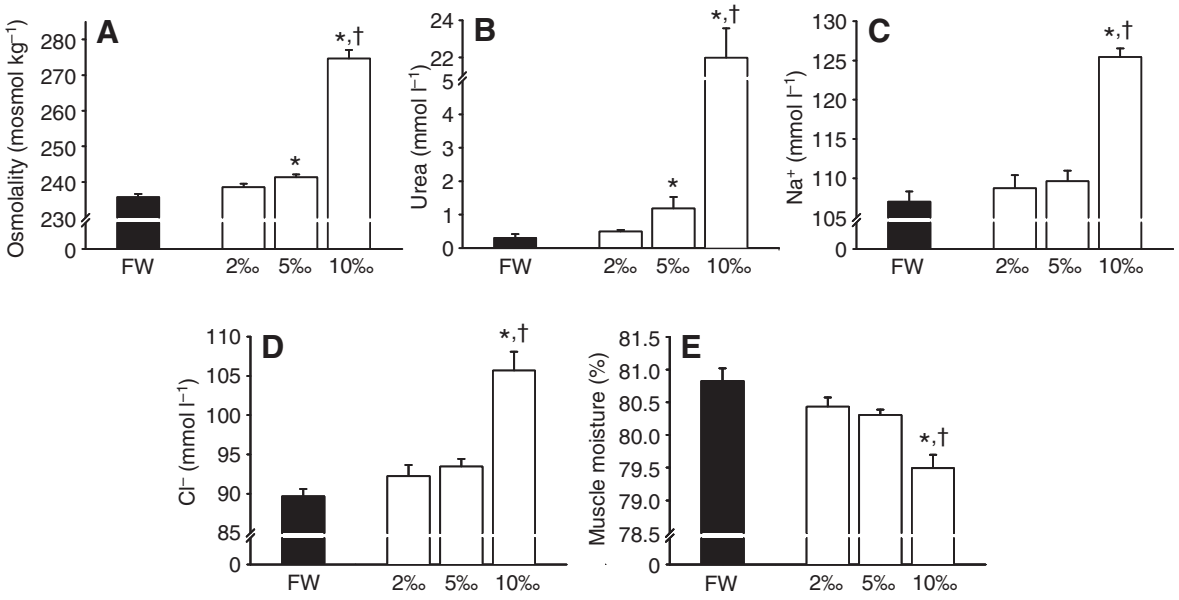

Fig. 4. The effects of brackish water (BW) acclimation on *Xenopus* serum (A) osmolality, (B) urea, (C) Na^+ and (D) Cl^- as well as (E) muscle moisture content. Data are expressed as mean values ± s.e.m., *N*=8-16 per group. *Significant difference ($P \leq 0.05$) from freshwater (FW) group. †Significant difference ($P \leq 0.05$) from all other BW-acclimated groups.

DISCUSSION

Overview

The current study demonstrates the presence of the integral TJ protein occludin in tissues involved in the maintenance of hydromineral balance in *Xenopus*. In response to BW acclimation, occludin mRNA expression alters significantly in key osmoregulatory epithelia (i.e. select regions of the GI tract, kidney and urinary bladder) and allows us to accept our hypothesis that, in *Xenopus*, mRNA abundance for the TJ protein occludin will respond to varying environmental salt concentrations in a tissue-specific manner. In tissues such as the kidney these alterations also occur in a salinity-dependent manner. The observed alterations in occludin mRNA expression generally fit with our current understanding of the physiological mechanisms that allow amphibians to maintain hydromineral balance in both FW and BW and we discuss our observations in the context of presumed alterations in paracellular permeability.

Occludin expression and localization in *Xenopus*

In general agreement with the relatively wide expression of occludin in other vertebrate groups (Furuse et al., 1993; Saitou et al., 1997; González-Mariscal et al., 2000; Ban et al., 2003; Acharya et al., 2004; Holmes et al., 2006; Laurila et al., 2007; Chasiotis and Kelly, 2008), occludin mRNA is broadly expressed in *Xenopus* tissues (Fig. 1). Amongst all organs examined, occludin mRNA expression appeared strongest in *Xenopus* rectum and urinary bladder (Fig. 1). These observations are consistent with high TER measurements across rectal and urinary bladder epithelia in amphibia, resulting in such tissues being classified as electrically 'very tight' (Claude and Goodenough, 1973; Krattenmacher and Clauss, 1988). Furthermore, occludin mRNA quantification in discrete regions of the GI tract of *Xenopus* revealed an increasing expression gradient along the longitudinal axis of the gut (Fig. 5A). This finding is also consistent with observations on the isolated intestine of *Rana esculenta*, where the colon (i.e. rectum) exhibited a higher TER than anterior regions of the GI tract (Saidane et al., 1997). Moreover, occludin immunolocalized within the *Xenopus* GI tract in a manner similar to patterns observed along the GI tracts of other vertebrates (Fig. 2) (Furuse et al., 1993; Inoue et al., 2006; Ridyard et al., 2007; Chasiotis and Kelly, 2008).

In the *Xenopus* kidney, occludin immunolocalized differentially in discrete regions of the nephron (Fig. 3). The pattern of localization appeared to parallel renal occludin immunostaining patterns in other vertebrates (Furuse et al., 1993; Kwon et al., 1998; González-Mariscal et al., 2000; Chasiotis and Kelly, 2008). More specifically, the presence of occludin immunostaining was observed in 'tighter' regions of the amphibian nephron (e.g. distal and collecting segments), where freeze-fracture analysis and TER measurements have revealed complex networks of several TJ strands and higher resistance, respectively (Brown, 1980; Taugner et al., 1982; Dantzler, 2003). The distribution pattern in *Xenopus* is notably similar to that of the freshwater goldfish (Chasiotis and Kelly, 2008). This is most likely because the two organisms have to address the same set of osmoregulatory problems, and the distal and collecting

Table 1. Effects of BW acclimation on Na^+,K^+-ATPase activity along the GI tract and in the kidney and urinary bladder of *Xenopus laevis*

		BW		
	FW	2‰	5‰	10‰
Stomach	0.57±0.07	0.98±0.07*	0.90±0.08*	0.77±0.08
Anterior intestine	2.14±0.10	2.04±0.26	2.61±0.19	2.15±0.13
Posterior intestine	1.18±0.12	0.88±0.10	0.83±0.11	1.04±0.05
Rectum	1.07±0.08	1.16±0.08	1.43±0.09*	1.51±0.07*
Kidney	6.51±0.21	5.45±0.28*	4.28±0.40*	4.78±0.30*
Urinary bladder	1.33±0.13	n.d.	n.d.	1.50±0.08

Na^+,K^+-ATPase activity is expressed as μmol ADP (mg protein)$^{-1}$ h^{-1} (mean values ± s.e.m., *N*=5–8 per group).
FW, fresh water; BW, brackish water; n.d., not determined.
*Significant difference ($P<0.05$) from FW group.

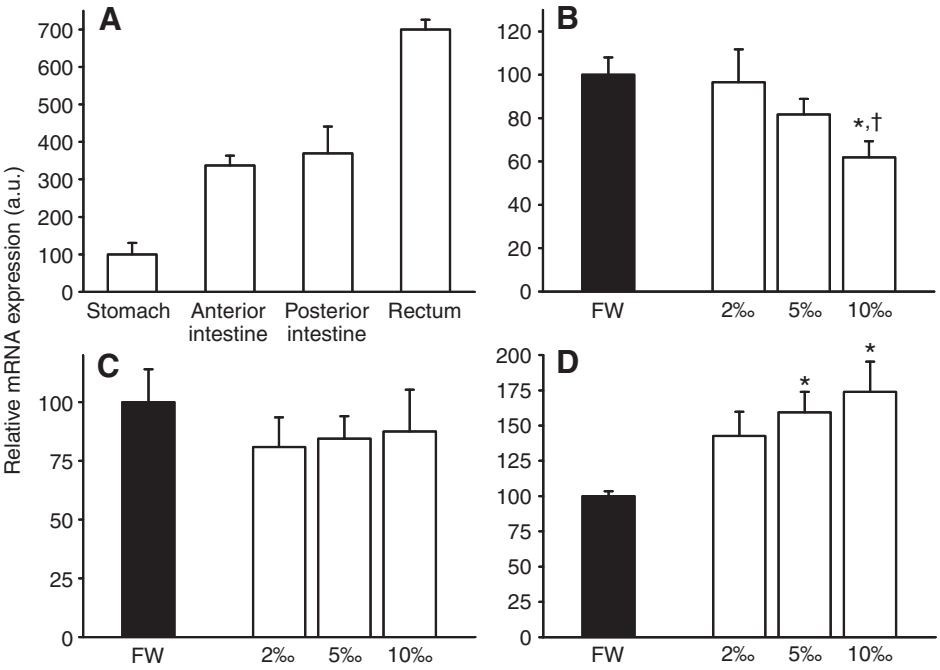

Fig. 5. (A) Occludin mRNA distribution in the *Xenopus* gastrointestinal (GI) tract by quantitative real-time PCR (qRT-PCR) analysis. Occludin mRNA expression increased along the longitudinal axis of the GI tract (i.e. stomach < anterior and posterior intestine < rectum). (B–D) The effects of BW acclimation on occludin mRNA expression in *Xenopus* (B) anterior intestine, (C) posterior intestine and (D) rectum. Occludin mRNA expression was normalized to β-actin mRNA expression (a.u., arbitrary units). Data are expressed as means ± s.e.m., N=6–7 per group. *Significant difference (P≤0.05) from FW group. †Significant difference (P≤0.05) from 2‰ BW-acclimated group.

segments of the non-mammalian aquatic vertebrate nephron are the primary sites of ion reabsorption (Dantzler, 2003).

Systemic endpoints of hydromineral balance in *Xenopus*

In the current study, *Xenopus* successfully acclimated to varying BW environments without mortality. However, salinity-dependent elevations in serum osmolality did occur, most likely caused by increased blood Na^+, Cl^- and urea concentrations (Fig. 4A–D). At the highest salinity (10‰), *Xenopus* appeared to maintain serum osmolality marginally lower than the surrounding water (i.e. ~275 mosmol kg^{-1} for serum *versus* ~300 mosmol kg^{-1} for 10‰ BW). Such phenomena have previously been documented in salt-acclimated frogs (Shpun et al., 1992). Therefore, while some tissue dehydration occurred (Fig. 4E), it would appear that urea accumulation in tissues may have reduced passive water loss to the environment, preventing critical dehydration (Jørgensen, 1997). In addition to acting as an osmolyte, urea and its accumulation in salt-acclimated amphibia is also believed to reflect an adaptive detoxification and elimination strategy for nitrogenous wastes (Janssens, 1964; Jørgensen, 1997). Accompanied by an up-regulation of enzymes involved with urea synthesis, normally ammoniotelic *Xenopus* adopts ureotelic strategies in BW, allowing toxic ammonia wastes to be converted into less toxic urea storage until environmental conditions favourable for ammonia excretion are restored (McBean and Goldstein, 1967; Janssens, 1972; Lee et al., 1982; Lindley et al., 2007).

The GI tract and BW acclimation in *Xenopus*

Dependent upon an electrochemical gradient generated by basolateral Na^+,K^+-ATPase, salt absorption across the amphibian intestine occurs through transcellular and paracellular routes (Nedergaard et al., 1999). Isolated colon from FW amphibians exhibits net Na^+ uptake (i.e. net Na^+ flux from mucosa to serosa) (Ferreira and Smith, 1968; Krattenmacher and Clauss, 1988). In contrast, isolated colon from saline-adapted amphibia exhibits net Na^+ secretion (i.e. net flux from serosa to mucosa) (Ferreira and Smith, 1968). Correspondingly, Na^+ levels in the colon faecal content of amphibia acclimated to saline conditions are significantly elevated relative to FW animals and typically exceed serum Na^+ levels (Ferreira and Smith, 1968; Ferreira and Jesus, 1973). In the current study, changes in occludin mRNA expression and Na^+,K^+-ATPase activity in the GI tract of BW-acclimated animals (Fig. 5; Table 1) seem to suggest that *Xenopus* utilizes the GI tract to cope with salt loading. Decreased occludin expression (and presumably

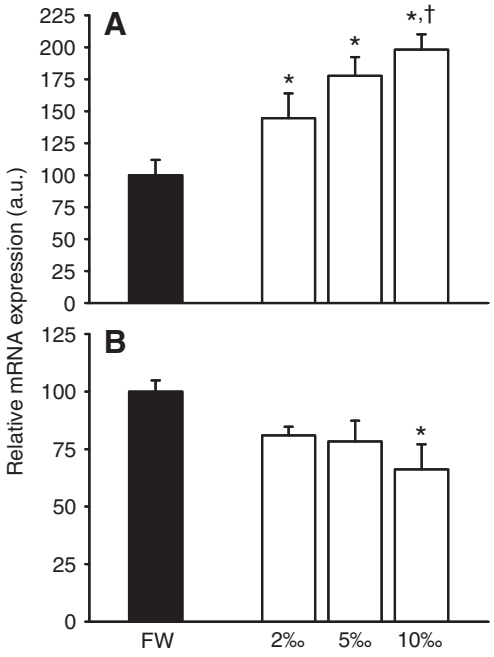

Fig. 6. The effects of BW acclimation on occludin mRNA expression in the *Xenopus* (A) kidney and (B) urinary bladder. Occludin mRNA expression was normalized to β-actin mRNA expression. Data are expressed as means ± s.e.m., N=4–6 per group. *Significant difference (P≤0.05) from FW group. †Significant difference (P≤0.05) from 2‰ BW-acclimated group.

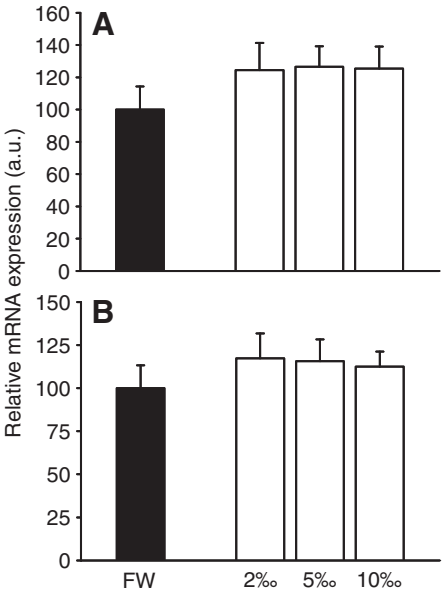

Fig. 7. BW acclimation and occludin mRNA expression in *Xenopus* (A) dorsal skin and (B) ventral skin. Occludin mRNA expression was normalized to β-actin mRNA expression. Data are expressed as means ± s.e.m., *N*=6–7 per group.

increased permeability) in the anterior intestine in response to BW would permit relatively greater movement of salt and water across this epithelium. Since the frogs are fed a diet of blood worms that have a high water content, a leakier anterior intestine would permit the passive diffusion of salts into the lumen of the intestine while the animals are feeding (Fig. 5B). However, increased occludin expression (and presumably decreased permeability) at the distal end of the GI tract (i.e. rectum; Fig. 5D) may allow elevating faecal salt levels to exceed those in serum, as previously observed in *Bufo* (Ferreira and Smith, 1968; Ferreira and Jesus, 1973). These strategies would be particularly useful because amphibians lack a loop of Henle in the kidney, and are thus unable to produce hyperosmotic urine. Therefore alterations in GI tract occludin expression may contribute to salt secretion across this organ system which, in turn, would play a significant role in limiting dehydration and salt loading in amphibia acclimated to saline conditions. These thoughts, however, are speculative and further evidence will require *in vitro* study of isolated regions of the GI tract in order to correlate occludin expression with measurements of epithelial 'tightness'.

The renal system and BW acclimation in *Xenopus*

In FW, the renal system of amphibians reabsorbs ions to combat obligatory ion loss to the surroundings. The bulk of ion reabsorption in the kidney takes place across epithelia of the distal nephron and the collecting duct (Dantzler, 2003). In these nephron segments, basolateral Na^+,K^+-ATPase activity establishes lumen-to-cell Na^+ gradients that facilitate Na^+ and Cl^- uptake. In the current study, we observed robust staining of Na^+,K^+-ATPase in the distal and collecting regions of the nephron (Fig. 3) and whole kidney Na^+,K^+-ATPase activity decreased in response to BW acclimation (Table 1). These overall changes in activity probably occur, at least in part, as a result of reduced enzyme activities in the distal and collecting regions and are in line with observations of reduced urine flow, decreased tubular salt re-uptake (i.e. natriuresis) and increased water reabsorption, leading to the excretion of small volumes of concentrated urine in BW-acclimated amphibians (Henderson et al., 1972; Shpun and Katz, 1995). We also observed an increase in occludin mRNA expression in the kidney in response to BW acclimation (Fig. 6A). Since occludin appears to be expressed in the same regions of the *Xenopus* nephron that exhibit abundant Na^+,K^+-ATPase expression, we contend that decreased epithelial permeability in these segments probably also contributes to reduced ion reabsorption. Support for this hypothesis is limited because the role of the paracellular pathway in ion reabsorption in non-mammalian vertebrates is not entirely known. However, in analogous segments of the mammalian nephron, paracellular Na^+ reabsorption contributes significantly to overall Na^+ recovery (Dantzler, 2003).

In order to avoid excessive hydration, FW amphibians excrete large volumes of dilute urine (Henderson et al., 1972; Shpun and Katz, 1995). Under such conditions, the epithelium of the urinary bladder is kept relatively 'tight' in order to prevent the passive flow of salts from hyperosmotic body fluids into the dilute contents of the bladder (Claude and Goodenough, 1973; Reuss and Finn, 1975). In saline conditions, the composition of ureteral urine changes substantially, with osmolality and solute concentrations increasing in accordance with environmental conditions (Shpun and Katz, 1995). Furthermore, a comparison of ureteral urine with urinary bladder urine collected from saline-adapted *Bufo* demonstrated that urine generated by the kidney is additionally subject to modification by the bladder, such that bladder urine can have a higher concentration of salts than ureteral urine (Shpun and Katz, 1995). While this could be the result of an increase in water reabsorption from the bladder, for example by increased expression of water channels (e.g. FA-CHIP) in saltwater-acclimated frogs (Verbavatz et al., 1992; Abrami et al., 1995), in our studies, decreased occludin expression in the *Xenopus* urinary bladder suggests that this epithelium also becomes 'leakier' under saline conditions (Fig. 6B). Since amphibian urine can be, at most, iso-osmotic with plasma, these data support the idea that salts may also be able to move into the bladder through the paracellular pathway (i.e. from serosa to mucosa). Indeed, isolated urinary bladders from *Bufo* bathed on the mucosal surface with increasing salt concentrations exhibit a reduction in TER and increased paracellular Na^+ flux into the bladder lumen independent from active transepithelial Na^+ transport (e.g. Na^+,K^+-ATPase) (DiBona and Civan, 1973; Reuss and Finn, 1975; Civan and DiBona, 1978; Finn and Bright, 1978). Accordingly, in our studies, Na^+,K^+-ATPase activity in the *Xenopus* urinary bladder did not significantly alter in response to BW acclimation (Table 1).

The integument and BW acclimation in *Xenopus*

When compared with other amphibians, *Xenopus* skin is relatively water impermeable and exhibits very low net active Na^+ uptake (Yorio and Bentley, 1978; Brown et al., 1981). Upon acclimation to saline conditions, *Xenopus* skin shows negligible changes in TER, Na^+ transport and Cl^- conductance, leading some authors to conclude that, unlike the skin of other amphibians, *Xenopus* skin does not play a key role in regulating salt and water balance (Katz and Hanke, 1993; Donna et al., 2004). Correspondingly, occludin mRNA expression in *Xenopus* dorsal and ventral skin did not significantly alter in response to salinity (Fig. 7).

Perspectives

The TJ complex plays an important role in amphibian hydromineral balance yet the role of TJ proteins in the regulation of epithelial permeability in this vertebrate group is poorly understood. Recent studies on FW fishes (e.g. Bagherie-Lachidan et al., 2008; Chasiotis and Kelly, 2008) point toward a dynamic role for TJs in the

maintenance of salt and water balance in aquatic vertebrates. In a FW environment, amphibians are faced with a similar suite of physiological problems to those of fishes and, to the best of our knowledge, the current study provides the first examination of amphibian TJ protein responses to environmental perturbation. Given the complexities of TJs and their properties, as well as the many challenges of an amphibious lifestyle, our understanding of the important role of the TJ complex and its protein 'machinery' in the physiology of amphibian homeostasis seems likely to grow with further investigation.

LIST OF ABBREVIATIONS

ADB	antibody dilution buffer
BW	brackish water
FW	freshwater
GI	gastrointestinal
qRT-PCR	quantitative real-time PCR
TER	transepithelial resistance
TJ	tight junction

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