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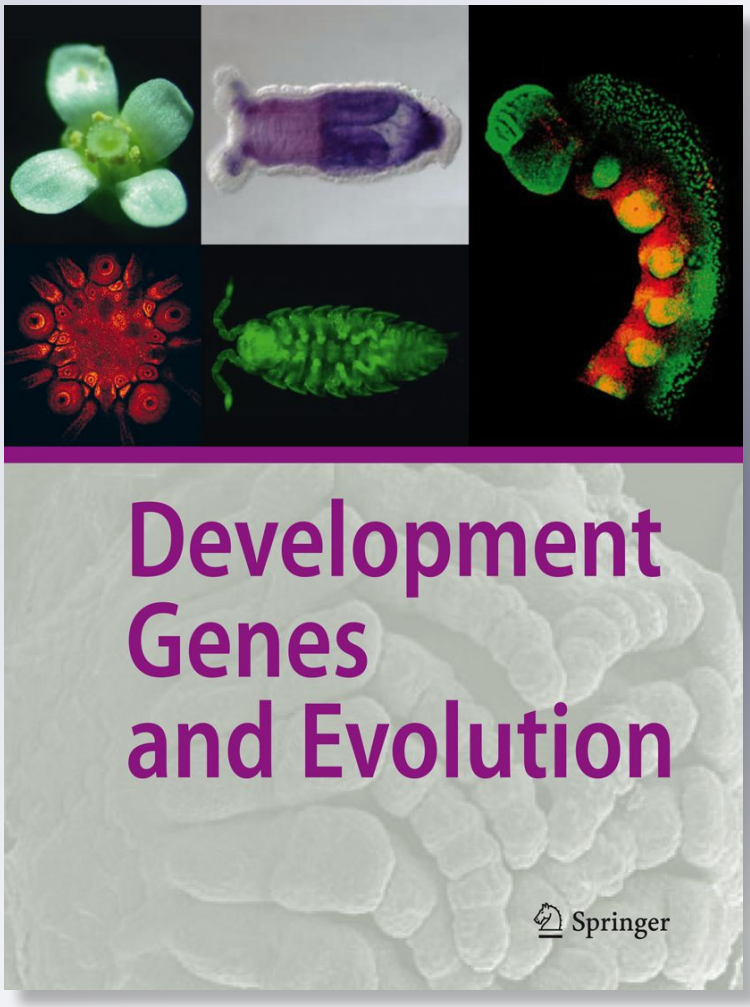

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SHORT COMMUNICATION

Evidence of a rudimentary colon in the elasmobranch, *Leucoraja erinacea*

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Abstract The transition from aquatic to terrestrial life presented tetrapodamorphs with the challenge of maintaining water homeostasis and preventing desiccation on land. The colon evolved in terrestrial vertebrates to help maintain fluid balance. Although marine elasmobranchs lack a colon, their spiral intestine contains a subregion that histologically appears to be colon-like, possibly representing an evolutionary precursor to terrestrial digestive tracts. The distal-most region of the spiral intestine of elasmobranchs has no villi and a large number of acid mucins: hallmarks of water absorption in the colons of terrestrial animals. To determine if histologically distinct regions of the elasmobranch digestive tract correspond to functional differences, we compared water absorption in different subregions of the skate, *Leucoraja erinacea* digestive tract. Water absorption in stomach and spiral intestinal sacs was linear with time and not hydrostatic pressure-dependent. The histologically distinct distal portion of the spiral intestine had a threefold higher rate of water absorption than the proximal portion of the spiral intestine. In addition, the water-selective, colon-specific aquaporin 4 is expressed strongly in the distal spiral intestine epithelia, correlating with the region of the spiral intestine exhibiting the greatest rate of water absorption. We demonstrate that the distal spiral intestine is histologically and functionally distinct from the rest of the spiral intestine and represents a rudimentary colon within the vertebrate lineage.

Keywords Digestive tract · Terrestrial evolution · AQP4 · *Leucoraja erinacea* · Acid mucins

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Introduction

Understanding the connection between developmental processes and morphological changes during evolution has been driven by vertebrate paleontology and developmental genetics. Particularly in limb evolution, comparisons of homologous gene expression patterns and functions have provided a clear understanding of the important mechanisms controlling initiation, outgrowth, and patterning of limbs (Abbasi 2011). In contrast, understanding the basis for morphological changes of visceral organs has depended upon comparative studies of gene expression and function in extant species. While much has been gleaned from this work, examination of the physiological importance of how tissues functioned over time has been mostly overlooked. In this paper, we begin to address the issue of "homology of function" of tissues; that as tissues and organs changed they still provided basic functions.

The acquisition of a colon is as important an event in tetrapod evolution as the fin-to-limb transition. In the aquatic to terrestrial transition, vertebrates contended with dehydration from evaporation and convection. To combat dehydration, terrestrial vertebrates evolved a colon to conserve water gained by drinking (Randall et al. 1997). The development of the colon allowed tetrapods to maintain water homeostasis by preventing desiccation and thus adapting to a terrestrial environment (Lacy 1991; Long and

Gordon 2004). The primary functions of the colon are to regulate water absorption and solute exchange. Characteristics that histologically distinguish the colon from the small intestine include the lack of villi and an elevation in acidic mucin-producing goblet cells (Filipe 1979). In terrestrial vertebrates, the presence of acid mucins is correlated with water absorption in the colon (Reifel and Travill 1979). Although a true colon is not believed to have evolved until the transition to terrestrial life, we have demonstrated that the skate *Leucoraja erinacea*, a cartilaginous fish, contains a region that histologically appears colon-like (Theodosiou et al. 2007). While much of the skate intestine contains elongated villi with low levels of acidic mucins, the distal-most region of the intestine lacks villi and contains a high number of acid mucins, similar to levels found in the colons of terrestrial vertebrates (Theodosiou et al. 2007). In addition, genes *Hoxa13* and *Hoxd13* that specify the colon are conserved in the skate, implicating posterior *Hox* gene function during development of the skate digestive tract (Theodosiou et al. 2007; Roberts et al. 1998; Warot et al. 1997). Based on this histology and molecular data, we proposed that the distal-most region of the spiral intestine of elasmobranchs (a subclass of the Class Chondrichthyes or cartilaginous fish) may have a rudimentary colon (Theodosiou et al. 2007).

The existence of a rudimentary colon in elasmobranchs is surprising because marine elasmobranchs have a unique mechanism of osmoregulation and they predate the emergence of tetrapodamorphs onto land. The body plasma of marine elasmobranchs is mostly isotonic to seawater due to high levels of urea and trimethylamine *N*-oxide in the plasma (Smith 1931). Thus, there is no apparent need for drinking and consequent water reabsorption in the digestive tract to maintain body plasma osmolality. Additionally, while cartilaginous fish appeared 450 million years ago, evidence of tetrapods inhabiting land occurred approximately 390 million years ago (Daeschler et al. 2006; Niedzwiedzki et al. 2010). A rudimentary colon in an elasmobranch suggests that either tetrapodamorphs were preadapted to conserve water before transitioning onto land, or elasmobranchs acquired this trait more recently.

While gene expression and function have been used to study homology in developing organisms, in this study, we examine the conservation of a visceral organ on a functional level. To examine homology of function, we investigated the ability of different regions of an elasmobranch digestive tract, *L. erinacea*, to absorb water. The net volume of water absorbed per length and surface area of tissue in different regions of the digestive tract is reported along with the rates of absorption (volume of water absorbed (in milligrams) per surface area (in square centimeters) per hour). In addition, subregions of the spiral intestine corresponding to previously shown histological differences were examined for differences in absorption rates. The pattern of expression of a water-selective aquaporin, aquaporin 4 (AQP4), is reported for subregions of the *L. erinacea* digestive tract.

Materials and methods

Animals

L. erinacea (common name, little skate) adult males were caught off the coast of Maine and kept in large outdoor flowing seawater tanks at the Mount Desert Island Biological Laboratory (MDIBL), Salisbury Cove, ME. Fresh tissues were obtained from 22 adult males. Animals were anesthetized in seawater containing 0.1 g/L tricaine and 0.035 g/L of $NaHCO_3$ and sacrificed by pithing. The MDIBL Animal Core Facility provided 26 euthanized animals. All procedures for animal use were in accordance with standards set by the NIH and approved by the institutional animal care and use committee at MDIBL.

Measurement of water absorption

To measure water absorption, we modified a protocol described for *Anguilla japonica* (Aoki et al. 2003). The stomach and spiral intestine were dissected and briefly rinsed with running seawater to remove intestinal contents and preincubated in elasmobranch Ringer solution (ERS) on ice (Forster et al. 1972). Digestive tract segments were filled to a pressure of 1.0 kPa with ERS, producing a ballooned stomach or intestinal "sac." To do this, a polyethylene tube (inner diameter, 0.125 in.; outer diameter 0.25 in.) was placed inside the proximal (rostal) end of the stomach or intestine and secured in place by cotton thread sutures. The tubing was connected to a flask filled with ERS that acted as a supply source for the ERS. A syringe attached to the flask was used to apply pressure and supply the organ with ERS. ERS was flushed through the tubing and tissue lumen to remove any air bubbles. Once air bubbles were removed, the distal (caudal) end of the organ was tied off and the tissue was filled with ERS, creating a ballooned sac, until a hydrostatic pressure of 1 kPa was obtained. A manometer (Manostar low differential pressure gauge WO81, Bellex International Corporation, Wilmington, DE), connected to the system by a Y-split, was used to monitor pressure inside the lumen. The tissue was detached by melting the tubing closed with flame-heated pliers and cutting through the seal. Gentle external pressure was applied to ballooned sacs to examine for any leaks before proceeding.

An initial length (in centimeters) and weight (in milligrams) of the ballooned sacs were recorded at 1 kPa before placing tissue in an aerated stirring bath of ERS. Sacs were removed from the ERS bath and blotted dry on the outside with Kimwipes™ to insure that the loss of mass was not due

to a leak in the sacs. The sacs were weighed several times and re-blotted if necessary until a consistent weight was obtained. Weights were recorded only when sacs weighed the same three times, measuring to 1/100th of a gram. The sacs were replaced in the ERS stir bath, and the blotting and weighing process was repeated every 30 min over a 3-h period. To examine regional differences, the spiral intestine was dissected into proximal (region closest to the stomach) and distal (region closest to the rectum) halves, excluding the rectum and rectal gland (Fig. 3a). To examine the effects of hydrostatic pressure on water absorption, intestinal sacs were brought to 1 kPa and then measured for length (in centimeters). The distal end of the tissue was sutured closed, and a final hydrostatic pressure of 1 or 3 kPa was adjusted before the tubing was sealed. Experiments were performed with each half of the intestine at 1 kPa and with whole intestinal sacs at 1 and 3 kPa, as described above. Fresh tissue was used in all experiments; one absorption experiment was performed on each digestive tract segment and the number of animals used per experiment is indicated in the respective figure legends.

Calculation of water absorption

The volume of water absorbed from the lumen across the gut epithelium and out into the stir bath was represented by the loss of mass of the ballooned sac over a given time interval. Thus, net water absorption was calculated as the change in mass at each time interval from time 0. The net water absorbed (in milligrams) per length (in centimeters) of tissue measured at 1.0 kPa is graphed (Fig. 1a). The effects of differences in surface area (SA) between the stomach and spiral intestine were examined in terms of net water absorbed in the organs (Fig. 1b). SA was determined after the water absorption measurements; the stomach was cut longitudinally, opened flat into a rectangle, and SA was measured. Likewise, the spiral intestine was cut open, laid flat, and the SA of the outer luminal rectangle was calculated. The internal folds of the spiral intestine opened as smaller rectangles whose SAs were measured and multiplied by 2, since both sides of the folds are covered in a villus epithelium (Theodosiou et al. 2007). Thus, the final SA equation of the spiral intestine was:

$$\mathrm{SA}_{\text{spiral intestine}} = (L \times W)_{\text{outer epithelium}} + 2(L \times W)_{\text{inner folds}} \times \text{the number of folds}$$

The rate of water absorption was documented as the volume of water absorbed per surface area (in square centimeter) per hour (Figs. 1a, b and 2). Only the first 2 h of data were used in calculating rates of absorption, as the volume of water absorbed over time appeared to plateau slightly after 2 h (Fig. 1a).

The final rate was calculated as:

$$\text{Rate} = (\text{mass}_{0\text{ h}} - \text{mass}_{2\text{ h}})/\text{SA}_{\text{Total}}/2\text{ h}.$$

Histology and immunohistochemistry

Previous work demonstrated that while the relative amounts of acid mucins increases from late embryonic to post-hatching animals in *L. erinacea*, the pattern and distribution of acid mucins are unchanged (Theodosiou et al. 2007). The increase of acid mucins post-hatching is likely the result of feeding and consequent increase in commensal bacterial, which can change the levels of mucosubstrates in the epithelium (Filipe 1979; Corfield et al. 1992). Since digestive tracts from late-staged embryos have a fully differentiated epithelium and are easier to handle than large adult digestive tracts, we used late-stage 30 skate embryos, which display swallowing in the egg case, for histology and immunohistochemistry studies (Ballard et al. 1993). *L. erinacea* embryos were obtained from the Marine Biological Laboratory (Woods Hole, MA) and procedures for the use of skate embryos were approved by the institutional animal care and use committee at Union College. Digestive tracts were harvested, fixed in 10 % formalin, and embedded in paraffin. Serial sections (6 μm) were cut and adjacent sections were stained for acid mucin-producing goblet cells, AQP expression, and antibody specificity controls, respectively (Fig. 3). To detect acidic mucosubstrates, sections were stained for 15 min with alcian blue (pH 2.5) and counterstained for 30 s with eosin–phloxine (Theodosiou et al. 2007).

To determine a role for aquaporins in the skate digestive tract, we chose AQP4. High-sequence identity among diverse species exists within the antigen region of commercially available polyclonal antibodies. AQP4 (Cat. #AB2218, Millipore) polyclonal antibody was tested for cross-reactivity with skate protein by dot blot analysis. For the dot blot, protein lysates were generated from the stomach, proximal and distal spiral intestine, and rectal gland. Respective tissues were homogenized in TG lysis buffer (20 mM HEPES, pH 7.2, 1 % Triton-X100, 10 % glycerol) containing 1 μg/ml aprotinin, 100 μg/ml PMSF, 1 μg/ml pepstatin, and 1:100 dilution of phosphatase inhibitor cocktail II (Cat. #P5725, Sigma-Aldrich) and centrifuged. The AQP4 antibody demonstrated cross-reactivity with lysate from the spiral intestine and rectal gland, but not the stomach (data not shown). To further demonstrate cross-reactivity of the rat polyclonal antibody with skate tissue, AQP4 polyclonal reactivity was blocked with a synthetic peptide for rat AQP4 on tissue sections (described below).

For AQP4 detection, paraffin sections were bleached in 0.5 % H_2O_2 for 30 min to inactivate endogenous peroxidase

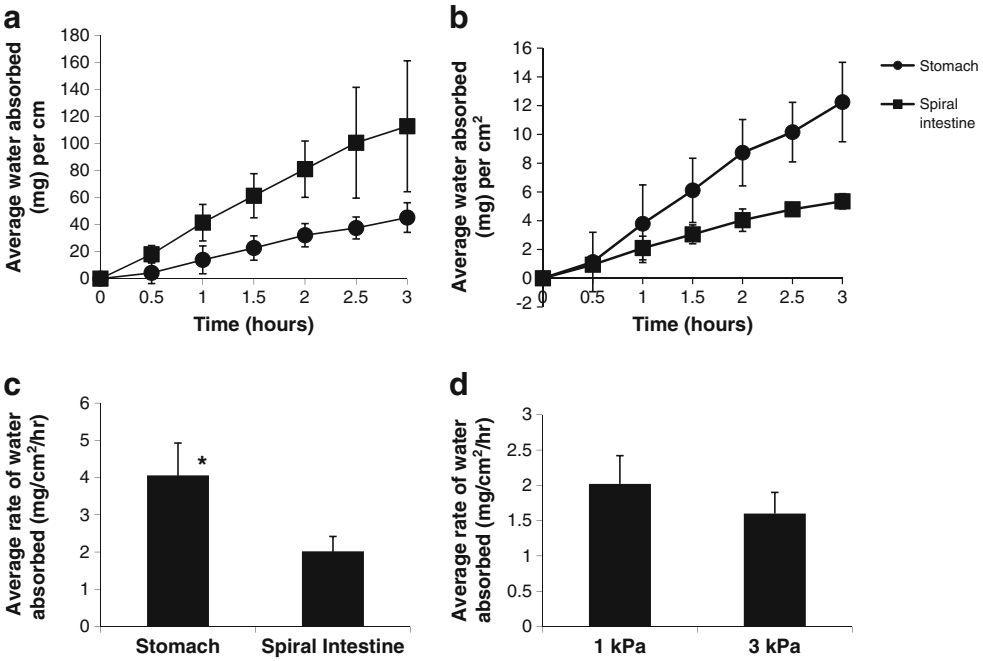

Fig. 1 Water absorption in the skate stomach and spiral intestine. **a** The net water absorbed (in milligrams of water) for each time interval to time 0 normalized for the length (in centimeters) of the organ is graphed. **b** Net water absorbed is normalized for the surface area (in square centimeters) of each organ (in **a**, **b**, $n=5$ for stomach, $n=5$ for spiral intestine). **c** The rates of water absorbed in stomach (4.06 mg/cm^2/h, $n=5$) and spiral intestine (2.02 mg/cm^2/h, $n=5$) are statistically different (*$P=0.019$). **a**–**c** Absorption experiments were performed at luminal pressures of 1 kPa. **d** No statistical difference exists between the rates of absorption at 1 kPa (2.02 mg/cm^2/h, $n=5$) and 3 kPa (1.6 mg/cm^2/h, $n=4$) ($P=0.13$)

activity and post-fixed in 4 % paraformaldehyde. To enhance antigen retrieval and diminish background, slides were boiled for 20 min in sodium citrate buffer (10 mM sodium citrate, 0.05 % Tween 20, pH 6.0) and allowed to cool to room temperature. The slides were blocked in 10 % horse serum and incubated overnight at 4 °C with rabbit anti-rat AQP4 at a 1:250 dilution in 1 % horse serum/PBT. The expression of AQP4 was visualized with a biotinylated-horse anti-rabbit 2° antibody (Vector Labs) at 1:250 in 1 % horse serum/PBT, followed by incubation in Vectashield ABC Elite (Vector Labs). Slides were developed in DAB mix for 20 min, dehydrated with xylene, and mounted in DPX permanent mountant. To test specificity of the antibody, a synthetic peptide from the C-terminus of the rat AQP4 (Cat. #AG777, Millipore) was preincubated in a 5:1 ratio with the rabbit anti-rat AQP4 polyclonal (1 % horse serum/PBT) for 12 h at 4 °C before proceeding with tissue incubation. No cross-reactivity was detected with the peptide inhibition assay (Fig. 3b″, c″, d″, e″). For negative controls, the slides were processed concurrently in which the primary antibody was omitted and found to have no staining (data not shown).

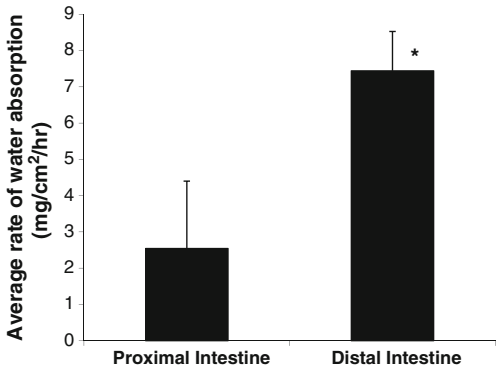

Fig. 2 Rates of water absorbed in the proximal and distal halves of the spiral intestine. *Asterisk*: Differences in absorption rates between the proximal (2.54 mg/cm^2/h, $n=4$) and distal (7.44 mg/cm^2/h, $n=4$) halves of the spiral intestine are significant ($P=0.013$)

Results and discussion

Differential water absorption in the *L. erinacea* digestive tract

Water absorption occurs in both the skate stomach and spiral intestine and is linear with time, higher in the spiral intestine than in the stomach per length (in centimeters) of tissue (Fig. 1a). However, the SAs (in square centimeters) of these organs differ greatly [stomach 22 cm^2 ($n=5$) and spiral intestine 140 cm^2 ($n=5$)]. When SA is considered, absorption is higher in the stomach (Fig. 1b). Consistent with this, the rate of water absorption (per square centimeters of SA per hour) is higher in the stomach (Fig. 1c, $P=0.019$). Thus, having a larger SA allows the spiral intestine to absorb more, and the stomach compensates the smaller surface area by having an increased absorption rate.

The solute composition of elasmobranch Ringer's solution used in our experiments was comparable to the ion concentrations reported for body plasma (Anderson et al. 2010; Forster et al. 1972). Water absorption in the isotonic conditions may reflect the mechanism that active ion

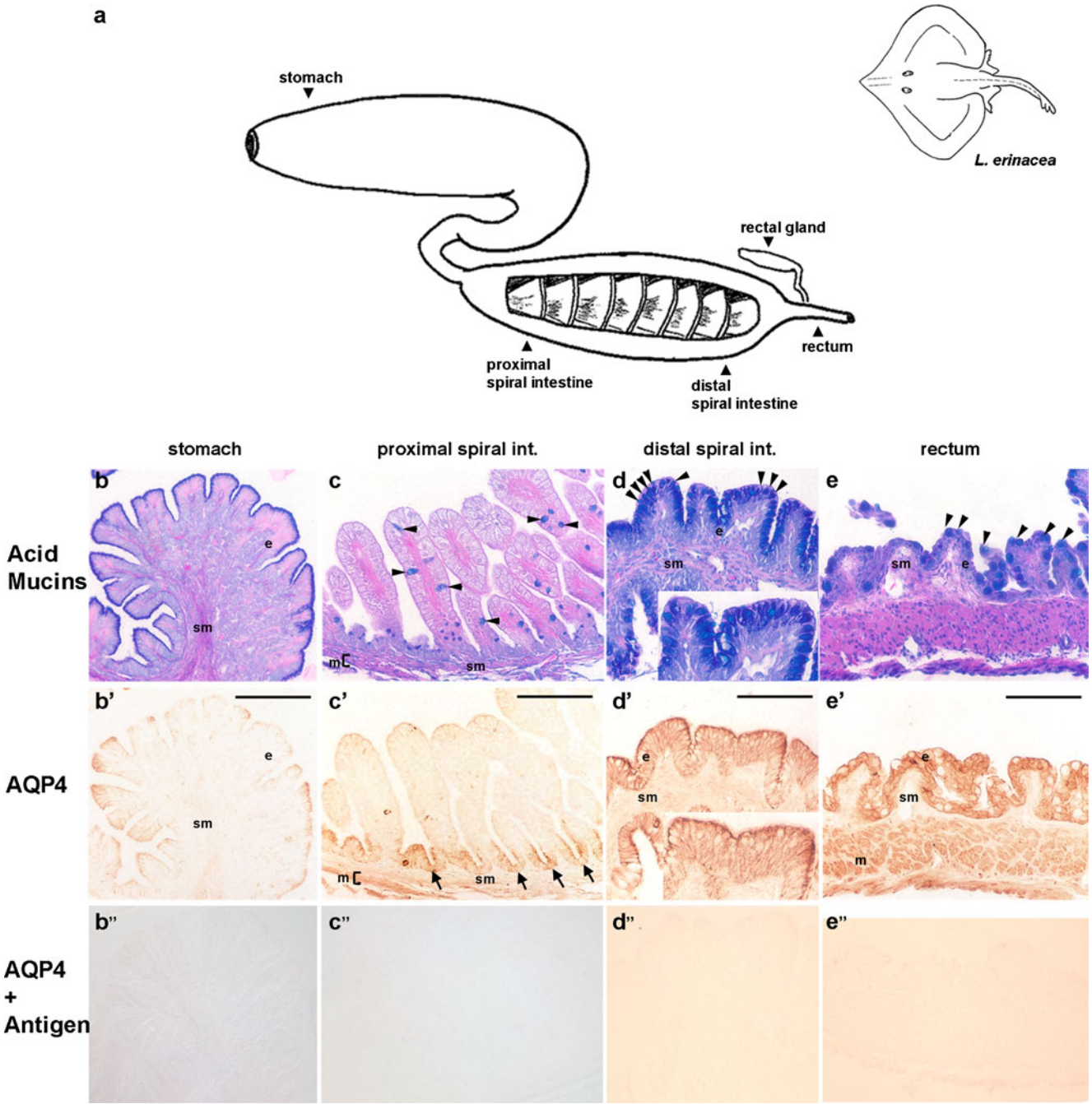

Fig. 3 AQP4 expression corresponds with the presence of acid mucin goblet cells in the *L. erinacea* digestive tract. **a** The anatomy of the *L. erinacea* digestive tract, with regions labeled, corresponding to panels **b**–**e** and Figs. 1 and 2 is illustrated. The spiral intestine is "windowed" to reveal the inner folds. **b**–**e** Alcian blue staining reveals the distribution of acid mucins in the digestive tract. Acid mucin-producing goblet cells are detected in the (**c**, *arrowheads*) proximal spiral intestine, (**d**, *arrowheads*) distal spiral intestine, and (**e**, *arrowheads*) cloaca, but absent from the (**b**) stomach. (*b′*–*e′*) Immunolocalization of AQP4 in adjacent sections to alcian blue stain (**b**-**e**). *Arrows* in *c′* highlight AQP4 expression in the crypts of the villi in the proximal spiral intestine. *d′*, *e′* AQP4 is expressed throughout the distal spiral intestine and rectum epithelia, respectively. **d**, *d′*, *inset* Higher magnification illustrates apical localization of AQP4 corresponds with cells containing acid mucin-producing goblet cells in the distal intestine. *c′*, *e′* AQP4 expression is detected in smooth muscle throughout the digestive tract. *b″*–*e″* Staining with AQP4 antibody inhibited by a C-terminus AQP4 synthetic peptide. *e* epithelium, *sm* submucosa, *m* muscularis. *Scale bars*, 100 μm

transport by the Na^+/K^+-ATPase drives water influx (Collie and Bern 1982; Oide 1967; Oide and Utida 1967). While not investigated here, it is likely that different salinities would affect the rates and volumes of water absorption. We examined if variations in luminal hydrostatic pressure, which in vivo could result from muscle contractions during peristalsis,

affected water absorption. During incubations of stomach and intestinal sacs, a decrease in volume would consequently lead to a decrease in hydrostatic pressure. No significant change in water absorption was observed in spiral intestinal sacs with luminal hydrostatic pressures of 1 and 3 kPa (2.02 and 1.6 mg/cm^2/h, respectively) (Fig. 1d). These data are consistent with work from coho salmon (Collie and Bern 1982). The failure of hydrostatic pressure to affect water absorption is in keeping with established models that active transport of solutes drives water flux in the skate intestine. In the future, this could be further confirmed by treatments with ouabain, an inhibitor of Na^+/K^+-ATPase (Oide 1967).

As our previous work demonstrated histologically distinct regions within the spiral intestine epithelium, we examined subregions of the spiral intestine for differences in absorption (Theodosiou et al. 2007). The distal half of the spiral intestine has a significantly higher rate of water absorption (7.44 mg/cm^2/h) than the proximal half of the intestine (2.54 mg/cm^2/h) with a P=0.01 (Fig. 2). Absorption rates are greater in the two halves than the whole spiral intestine because the SAs are reduced; when cut in half and ballooned sacs are made, the luminal folds are disrupted and the SA of the halves decreases relative to the total SA of the whole intestine (Fig. 1c). The increased rate of absorption in the distal spiral intestine compared to the proximal half is consistent with the higher rate of absorption observed in the distal (posterior) intestine of the Japanese eel (Aoki et al. 2003). Significant differences in rates of water absorption in histologically distinct regions of the spiral intestine suggest that within the spiral intestine, there may be different mechanisms for regulating water absorption.

AQP4 expression in *L. erinacea* digestive tract

The water-selective AQP4 is expressed in surface epithelial cells and functions to absorb water in the mammalian colon (Wang et al. 2000; Ma and Verkman 1999). We investigated whether aquaporin isoforms may be implicated in the mechanisms for water absorption in the skate. The AQP4 polyclonal demonstrated cross-reactivity with skate protein from spiral intestine and rectal gland, but not stomach lysate on dot blot (data not shown). A synthetic peptide for the C-terminus of rat AQP4 blocked reactivity of AQP4 rat polyclonal against skate tissue (Fig. 3b″–e″). Thus, we proceeded with analysis of AQP4 expression in the different subregions of the skate digestive tract.

In the *L. erinacea* stomach, AQP4 is not reproducibly or uniformly expressed above background in the mucosal epithelium, corresponding to an epithelium that does not contain goblet cells (Fig. 3b, b′). AQP4 expression is specific in the endoderm-derived epithelium of the spiral intestine. In the proximal half of the spiral intestine, AQP4 is localized to the crypts of villi where there are few acid mucin-producing goblet cells (Fig. 3c, c′). Colonic crypts are the major site of fluid absorption (Welsh et al. 1982; Naftalin 1994; Singh et al. 1995). AQP4 crypt expression in skate suggests that aquaporins may be conserved during digestive tract evolution.

In the distal spiral intestine, AQP4 expression is strongly expressed in the apical epithelium where acid mucins are abundant in adjacent sections (Fig. 3d, d′). The expression of AQP4 remains strong in the rectum epithelium where the numbers of acid mucin-producing goblet cells decrease (Fig. 3e, e′). Throughout the digestive tract, AQP4 was absent from the submucosa, but present in the muscularis (Fig. 3c′, e′). The increased AQP4 expression corresponds with the higher rate of water absorption found in the distal spiral intestine (Fig. 2). The correlation between relative levels of AQP4 expression and water absorption between subregions of the digestive tract suggests that an aquaporin-based transcellular pathway may be the mechanism for water absorption in the skate digestive tract.

Implications for colon evolution

While evolution and development studies have focused on gene expression studies to identify the homology of tissues, this study determines if homologous tissues and organs can still provide the same basic functions. In this study, we report that regional histological differences in the *L. erinacea* digestive tract reflect functional differences in rates of water absorption. The correlation of colon cell markers with differential water absorption in the distal spiral intestine of an elasmobranch suggests several models for colon evolution. First, the rudimentary colon in the elasmobranch may be analogous to the tetrapod colon, having arisen by convergent evolution. Studies have demonstrated that elasmobranchs do drink in response to changing salinities or induced hypovolemia (Anderson et al. 2002; De Boeck et al. 2001; Hazon and Henderson 1984). This may have allowed elasmobranchs to adapt to different environments with changing salinities. Of modern elasmobranchs, 5 % are euryhaline and can inhabit both fresh and salt water (Ballantyne and Robinson 2010). Another possibility is that despite their method of osmoregulation, the elasmobranch digestive tract was preadapted for conserving water prior to the tetrapod transition. This is supported by similar epithelial histology and acid mucin distribution in the distal spiral intestine of *Squalus acanthias* (Theodosiou, unpublished). In addition, *A. japonica* has an increased the rate of water absorption in the distal intestine (Aoki et al. 2003). While it is unlikely that the digestive tract evolved in parallel in such divergent species, if a rudimentary colon predates the tetrapod transition to land, this would suggest that the colon in

terrestrial vertebrates evolved from an expansion of a rudimentary colon in elasmobranchs. Differential water absorption in the spiral intestine may have conferred a selective advantage for ancestral cartilaginous fish to allow invasion of water habitats with varying salinities as a prelude to terrestrial invasion. We have shown through histology, gene expression, and now physiology that the skate's distal spiral intestine is a rudimentary colon-like structure that may be the evolutionary precursor to the tetrapod colon.

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