Morphology and Putative Function of the Colon and Cloaca of Marine and Freshwater Snakes

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ABSTRACT Among tetrapods, evidence for postrenal modification of the urine by the distal digestive tract (including the colon and cloaca) is highly variable. Birds and bladderless reptiles are of interest because the colon and cloaca represent the only sites from which water and ions can be reclaimed from the urine secreted by the kidney. For animals occupying desiccating environments (e.g., deserts and marine environments), postrenal modification of the urine may directly contribute to the maintenance of hypo-osmotic body fluids. We compared the morphology and distribution of key proteins in the colon, cloaca, and urogenital ducts of watersnakes from marine (*Nerodia clarkii clarkii*) and freshwater (*Nerodia fasciata*) habitats. Specifically, we examined the epithelia of each tissue for evidence of mucus production by examining the distribution of mucopolysaccharides, and for evidence of water/ion regulation by examining the distribution of Na^+/K^+-ATPase (NKA), $Na^+/K^+/Cl^-$ cotransporter (NKCC), and aquaporin 3 (AQP3). NKCC localized to the basolateral epithelium of the colon, urodeal sphincter, and proctodeum, consistent with a role in secretion of Na^+, Cl^-, and K^+ from the tissue, but NKA was not detected in the colon or any compartment of the cloaca. Interestingly, NKA was detected in the basolateral epithelium of the ureters, suggesting the urothelium may play a role in active ion transport. AQP3 was detected in the ureters and coprodeal complex, consistent with a role in urinary and fecal dehydration or, potentially, in the production of the watery component of the mucus secreted by the coprodeal complex. Since no differences in general cloacal morphology, production of mucus, or the distribution of ion transporters/water channels were detected between the two species, cloacal osmoregulation may either be regulated by proteins not examined in this study or may not be responsible for the differential success of *N. c. clarkii* and *N. fasciata* in marine habitats. J. Morphol. 273:88–102, 2012.

KEY WORDS: aquaporin 3; cloaca; colon; Na^+/K^+-ATPase; $Na^+/K^+/2Cl^-$ cotransporter; Nerodia

INTRODUCTION

The renal concentrating capacity of the reptilian kidney is known to be poor (Braun, 1998). Despite this, several groups of reptiles are capable of varying the concentration of their urinary waste through postrenal modification of the waste products in the bladder (turtles, tuataras, and some lizards) or, in bladderless reptiles (crocodiles, snakes, and some lizards), in the colon/cloaca (Dantzler and Bradshaw, 2009). Schmidt-Neilsen et al. (1963) hypothesized that animals possessing functional salt glands may increase reabsorption of salt from the distal digestive tract (colon/cloaca) during times of salinity acclimation to gain water via solute-linked water reabsorption. Comparisons of cloacal urine composition following salinity acclimation in *Crocodylus porosus* (marine) and *Alligator mississippiensis* (freshwater) support this hypothesis (Pidcock et al., 1997), however, studies of lizards (Bradshaw and Shoemaker, 1967) and tortoises (Nagy and Medica, 1986) reveal an alternative osmoregulatory strategy in desert environments. These latter studies suggest that even species without a salt gland may benefit from solute-linked reabsorption of water if they can tolerate the associated increase in plasma ion concentrations during intermittent times of drought. To our knowledge, only two previous studies have explicitly examined the putative osmoregulatory function of the colon/cloaca in snakes (Seshadri, 1959; Junqueira et al., 1966). Using histology and analysis of urine electrolytes, these studies suggest that the colon/cloaca play a role in postrenal modification of the urine through reabsorption of Na^+ (Junqueira et al., 1966) and water (Seshadri, 1959; Junqueira et al., 1966). Importantly, these previous studies examined only terrestrial species while the putative physiology of the gut/cloaca in aquatic snakes is unknown. Additionally, the distribution of ion transporters and water channels has never

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been examined in the gut/cloacal tissues of any snake species.

Although modification of the urine has been shown to occur in the coprodeum (Schmidt-Nielsen and Skadhauge, 1967) and urodeum (Kuchel and Franklin, 2000) of crocodilians, among lizards retrograde flow of the urine into the colon suggests that the intestinal epithelium may also be an important site of ion and water reclamation (Bentley and Bradshaw, 1972; Skadhauge and Duvdevani, 1977). Despite differences in habitat use and diet, many reptile species produce cloacal urine (i.e., urine collected after modification) that is low in Na^+ and high in K^+, relative to the ureteral urine (Bentley and Schmidt-Nielsen, 1965; Schmidt-Nielsen and Skadhauge, 1967; Minnich, 1970; Robinson and Dunson, 1976; Skadhauge and Duvdevani, 1977; Taplin, 1985). Additionally, the relative concentration of these ions is known to vary with dehydration and salt loading in some species (Bradshaw, 1972, 1975; Skadhauge and Duvdevani, 1977; Bradshaw and Rice, 1981; Kuchel and Franklin, 1998). The mechanisms by which this variation in ion secretion and water reabsorption might occur have received little attention in reptiles (but see: Bentley and Bradshaw, 1972, and references therein).

Here, we examine the morphology and immunohistochemistry of the colon, cloaca, and urogenital ducts of closely related marine and freshwater watersnakes to determine if evidence for secretion or reabsorption of Na^+ and water exists in aquatic species, and if the distribution of ion transporters and water channels differs between freshwater and marine species. In a previous study we have shown that after acclimation to 0, 50, and 100% seawater (SW), plasma osmolality remains low in the saltmarsh snake *Nerodia clarkii clarkii* and increases with salinity in the freshwater *Nerodia fasciata* (Babonis et al., in press). *Nerodia fasciata* has also been shown to have reduced survival in seawater (even over very short time periods) as compared with its marine congeners (Babonis, et al., in press; Pettus, 1963; Dunson, 1980). Thus, if the osmoregulatory capability of the cloaca is at least partly responsible for enabling *N. c. clarkii* to tolerate marine habitats, then, when acclimated to seawater, we expect *N. c. clarkii* to block the NaCl reabsorption pathway to prevent increased plasma ion concentrations. By contrast, *N. fasciata*, a snake that inhabits freshwater habitats, is not expected to have this response to increasing salinity. Because the *N. clarkii* and *N. fasciata* complexes are likely sister taxa (Lawson et al., 1991), we find comparisons of subspecies from these two taxa, which utilize very different habitats, to be particularly useful in attempting to understand the evolution of marine habitat use among snakes.

To test the hypothesis that the cloaca may be partly responsible for differences in salinity tolerance between *N. c. clarkii* and *N. fasciata*, we examined the effect of salinity acclimation on the morphology of the colon/cloaca as well as the distribution of two ion transporters, Na^+/K^+-ATPase (NKA) and $Na^+/K^+/Cl^-$ cotransporter (NKCC), and one water channel, aquaporin 3 (AQP3), in these tissues. NKA is an enzyme that is expressed nearly ubiquitously in the basolateral membranes of vertebrate tissues where it facilitates the asymmetrical exchange of Na^+ and K^+, a process thought to be critical in the regulation of cell homeostasis. When NKA is co-expressed in the basolateral membrane with NKCC (isoform 1), these proteins facilitate the net secretion of NaCl, as is the case in many secretory epithelia (Haas and Forbush, 2000). By contrast, when basal NKA is co-expressed with apical NKCC (isoform 2), net Na^+ transport is in the opposite direction; this process mediates net Na^+ reabsorption in the mammalian kidney (Kinne and Zeidel, 2009). Since Na^+ reabsorption is often tightly linked to water reabsorption, we also examined the distribution of AQP3, a water channel common in the mucosecretory epithelia of the digestive tract in other vertebrates (Matsuzaki et al., 1999; Lignot et al., 2002; Pandey et al., 2010). Though NKA, NKCC1, and AQP3 are known to be distributed widely in snake tissues (including some components of the digestive tract; Babonis et al., in press;), their potential role in regulating Na^+ and water balance in the colon, cloaca, and urogenital ducts of watersnakes from different habitats have not been examined.

Consistent with the previous observation of active Na^+ reabsorption in the cloaca of snakes, we expect to find a basolateral NKA and an apical NKCC in the colon/coprodeum and in the urodeum of the cloaca of both species. Additionally, if the cloacal water reabsorption hypothesized by Junqueira et al. (1966) and Seshadri (1959) is facilitated by aquaporin-mediated transepithelial water flux, we expect to find a basolateral localization of AQP3 as well. Finally, if differential regulation of cloacal water/ion transport is partly responsible for the ability of *N. c. clarkii* to survive in marine environments, we expect different responses to salinity acclimation in *N. c. clarkii* and *N. fasciata*. Because both the localization and abundance of NKA and NKCC are known to vary with acclimation to salinity in some euryhaline fishes (reviewed by: Evans and Claiborne, 2009), we expect to find changes in the localization and/or abundance of these proteins in *N. c. clarkii* following acclimation to 100% seawater (SW), as compared to those individuals acclimated to 0% SW. Specifically, we expect a decrease in the abundance of NKA and NKCC in the colon/cloaca of *N. c. clarkii* following acclimation to high salinity, which would be consistent with a reduction in the reabsorption of Na^+ from these tissues. Likewise, if *N. c. clarkii* experiences water reabsorption even following acclima-

tion to 100% SW, we expect the localization and/or abundance of AQP3 to be invariant in this species. Furthermore, if cloacal ion and water transport is related to habitat use, we expect that *N. fasciata* might lack this plasticity in cloacal ion transporter distribution and/or abundance. Because the morphology and histochemistry of the colon, cloaca, and urogenital ducts of *N. c. clarkii* have not yet been described, we also provide a detailed description of the epithelia in each of the examined tissues, with specific reference to cell shape (which is often associated with secretory potential) and the presence of mucus.

MATERIALS AND METHODS

Animal Collection and Maintenance

Gulf coast salt marsh snakes (*N. c. clarkii*) and banded watersnakes (*N. fasciata*) were collected from Levy and Alachua counties (FL), respectively, during the spring/summer of 2007. Animals were returned to the University of Florida and housed individually in plastic aquaria containing enough freshwater (tapwater, Gainesville, FL) to completely cover their cutaneous surfaces as they rested on the bottom. All animals were acclimated to laboratory conditions for a period of 5 days in 0% SW. After the 5-day acclimation period, control animals were selected randomly ($N = 5$ from each species of *Nerodia*) and sacrificed via rapid decapitation. Remaining animals were then randomly assigned to one of the following treatments ($N = 5$ per treatment, per species): 0, 50, or 100% SW and acclimated to their final salinity by incremental salinity increases over a period of 7 days. Animals were then retained in their final salinity for an additional week while still receiving daily cage water changes (tapwater or salt water mixed from Instant Ocean and tapwater). At the end of this experimental period, all animals were sacrificed via rapid decapitation in accordance with the American Veterinary Medical Association's guidelines on euthanasia using methods approved by the University of Florida's Institutional Animal Care and Use committee.

Tissue Collection and Preservation

Tissues were removed from animals immediately following sacrifice, rinsed of excess blood and fecal/urinary waste using 10 mmol l^{-1} phosphate buffered saline (PBS), and fixed immediately in 4% paraformaldehyde (diluted in deionized water) at 4°C for 24 h. Following fixation, tissues were rinsed in three washes of 10 mmol l^{-1} PBS (15 min each) and stored in 75% ethanol overnight at room temperature (RT). Tissues were then transferred to a fresh aliquot of 75% ethanol where they were stored, at RT, until processing. Before embedding, tissues were dehydrated through a series of ethanol baths of increasing concentration, followed by two 1 h rinses in Citrisolv (Fisher Scientific, Pittsburgh, PA), and four changes of paraffin wax (Tissue Prep 2, Fisher Scientific) at 55°C for 1 h each. Embedded tissues were sectioned at 7 μm, mounted on charged glass slides (Superfrost Plus, Fisher Scientific), and dried overnight at 30°C.

Histology and Immunohistochemistry

The basic structure of the epithelia and supporting tissues for the colon and cloacal chambers was viewed using the Lillie modification of the Masson Trichrome stain (Humason, 1972). Acid mucins were detected using Alcian blue (pH 2.5; Humason, 1972) and the presence of neutral mucins is inferred from the presence of periodic acid Schiff positive (PAS^+) reaction (Humason, 1972). To examine the distribution of NKA, NKCC, and AQP3 in the epithelia, rehydrated tissue sections were incubated overnight at 4°C in anti-NKA (1/100), anti-NKCC (1/2000) or anti-AQP3 (1/100; these dilutions were previously optimized for use in these species/tissues and applied to adjacent sections on the same or, when necessary, adjacent slides) following a 30 min peroxidase block and a 20 min protein block (BioGenex, San Ramon, CA), both at RT. Protein localization was then visualized using the Super Sensitive™ Link-Label universal secondary antibody kit (BioGenex) and 3'3-diaminobenzidine chromagen (BioGenex). Negative controls were produced in a similar manner by incubating adjacent sections in BioGenex Protein Block rather than primary antibody. Images were produced using a Hitachi KP-D50 digital camera (Hitachi, Tokyo, Japan) mounted on an Olympus BX60 light microscope (Olympus, Center Valley, PA), digitized using ImagePro Express software (Media Cybernetics, Bethesda, MD) and brightened using Adobe Photoshop CS3 (San Jose, CA). A minimum of three individuals were examined for each species/treatment.

Primary Antibodies

Monoclonal anti-NKA (α5), developed by Dr. Douglas Fambrough, and monoclonal anti-NKCC (T4), developed by Drs. Christian Lytle and Bliss Forbush III, were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. Anti-NKA is directed against the α1 subunit of the NKA heterodimer (Takeyasu et al., 1988). Anti-NKCC is directed against a conserved epitope in the carboxyl tail of NKCC1, NKCC2, and NCC (Lytle et al., 1995). Anti-AQP3 (Hc-3) is directed against the following epitope in the c-terminus of treefrog AQP3: CQENVKLSNVKHKERI (Pandey et al., 2010). Hc-3 and its blocking peptide were generous gifts from Dr. David Goldstein at Wright State University. Antibody specificity has previously been confirmed via Western blotting (NKA and NKCC) or by peptide preabsorption (AQP3; Babonis et al., in press).

RESULTS

Morphology of the Colon and Cloaca

Here, we use the terminology of Siegel et al. (2011a) to describe the tissues comprising the cloaca of watersnakes. Specifically, we use the term "colon" to describe the most posterior portion of the large intestine, and use the term "coprodeal complex" as inclusive of both the colon and the urodeal sphincter. Colon samples were collected from the portion of the intestine just posterior to the ileocecal valve, which separates the small and large intestine. This portion of the intestine was easy to identify in both species because it frequently held a bolus of semi-solid fecal waste in contrast with the posterior-most segment of the small intestine, which was always empty. The position of the colon relative to the cloaca (and the rest of the urogenital organs) is illustrated in Figure 1. The cloaca of watersnakes is composed of three main chambers: i) the coprodeum, which is synonymous with the colon, ii) the urodeum, which comprises two short finger-like chambers projecting toward the anterior, hereafter referred to as the left and right (L/R) urodeal chambers, and a common chamber into which the L/R urodeal chambers empty, and iii) the proctodeum, which

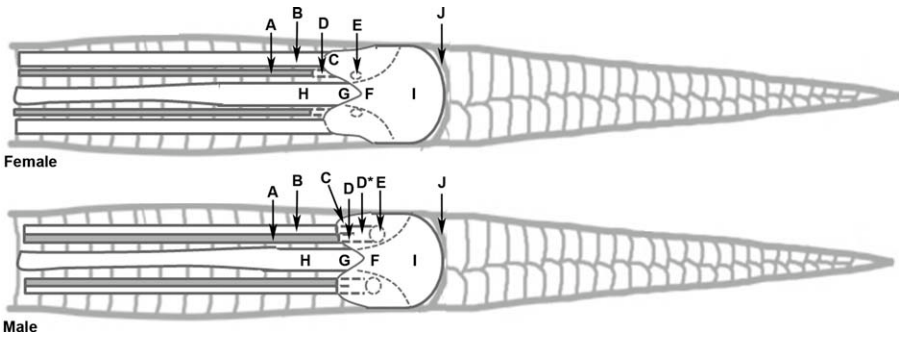

Fig. 1. Relative positions of cloacal chambers in female (upper) and male (lower) watersnakes. The anterior of the animal is to the left in this figure. A, ureter; B, posterior vagina (female)/ductus deferens (male); C, L/R urodeal chambers; D, ampulla uriniferous papilla (female)/ampulla urogenital papilla (male); D*, junction of the ductus deferens and ampulla urogenital papilla; E, junction of the ampullae (female and male) with common urodeal chamber; F, common urodeal chamber (dotted lines indicate coprodeal/urodeal junction); G, urodeal sphincter; H, colon; I, proctodeum; J, vent. Snake body outline adapted from: http://www.reptilesdownunder.com/arod/scale/.

communicates anteriorly with the common urodeal chamber and posteriorly with the vent (Fig. 1).

The colonic epithelium of both species (Fig. 2A–C) is a simple columnar epithelium dominated by cup-shaped goblet cells (Gc) interspersed with tall columnar enterocytes (Et). The basement membrane (bm) is supported by a highly vascularized (red blood cells marked by *) lamina propria (lp). Supporting the lamina propria is a loosely organized submucosa (sm) surrounded by a layer of circular muscle (cm), a small amount of connective tissue (ct), and a transverse muscle (tm) layer. Where the colon communicates with the urodeal sphincter the epithelium becomes more pseudostratified with dark-staining basal cytoplasm and elongate basally-to-centrally positioned nuclei (Fig. 2D–F). Posteriorly, the epithelium of the coprodeal complex becomes pseudostratified and the clear-staining apical cytoplasm becomes reduced in size such that the cells nearest the colon are columnar in shape (Fig. 2E) whereas those near the junction with the urodeum are more cuboidal (Fig. 2F). The frequency of goblet cells decreases as the coprodeal complex progresses posteriorly toward the proctodeum. The L/R urodeal chambers are located dorsolaterally to the coprodeal complex in both species. In females, the L/R urodeal chambers communicate with the posterior-most ends of the vaginal pouches; in males, the L/R urodeal chambers are shorter (rostrocaudally) but otherwise similar in morphology. The epithelium of the L/R urodeal chambers is simple or slightly pseudostratified with tall columnar cells that have clear-staining cytoplasm (Fig. 2G). There is a brush border lining the mucosal membranes of these chambers, which becomes patchy in the posterior. Moving caudally, the L/R urodeal chambers merge along their midline to form the common urodeal chamber (Fig. 2H), which is typified by a low pseudostratified epithelium. The cells of the anterior-most portion of the common urodeal chamber are very tall columnar cells, which decrease in height toward the posterior (Fig. 2I). The low cuboidal urodeum merges with the coprodeal complex to form the anterior boundary of the proctodeum (Fig. 2J), a transition marked by a decrease in cell height such that the epithelium of the proctodeal chamber transitions into nonkeratinized stratified squamous epithelium, anteriorly (Fig. 2K), and keratinized stratified squamous epithelium as this chamber approaches the vent (Fig. 2L).

The ducts of the reproductive tract as well as the ureters can easily be seen in the supporting tissue around the cloaca in these species. In females, the posterior vagina (V), the most posterior portion of the oviduct, can be easily distinguished from other ducts in the urogenital region by the extremely thick circular muscle layer surrounding it (shown relative to the thickness of the ureter in Fig. 3A). Inside the circular muscle layer is a thick submucosa and a lamina propria directly underlying the simple columnar epithelium of the vagina. A brush border (bb; Fig. 3B) can be seen on the apical membrane of the vaginal epithelium. The left and right posterior vaginas transition, posteriorly, into the left and right urodeal chambers (Fig. 3C), a transition that is marked, most notably, by a decrease in the thickness of the circular muscle layer around the L/R urodeal chambers (compare the thickness of the submucosa of the vagina and urodeum in Fig. 3C). This supporting tissue also stains lighter with Trichrome (Fig. 3C) than does the supporting layer around the vagina (V). The epithelium of the L/R urodeal chambers is very similar to that of the vaginal epithelium except that the urodeal epithelium appears to be more pseudostratified (compare Fig. 2H with Fig. 3B), and the brush border is less obvious than in the vagina.

In males, the ductus deferens is situated laterally to the other urogenital organs, cranially, and communicates with the urodeum caudally through

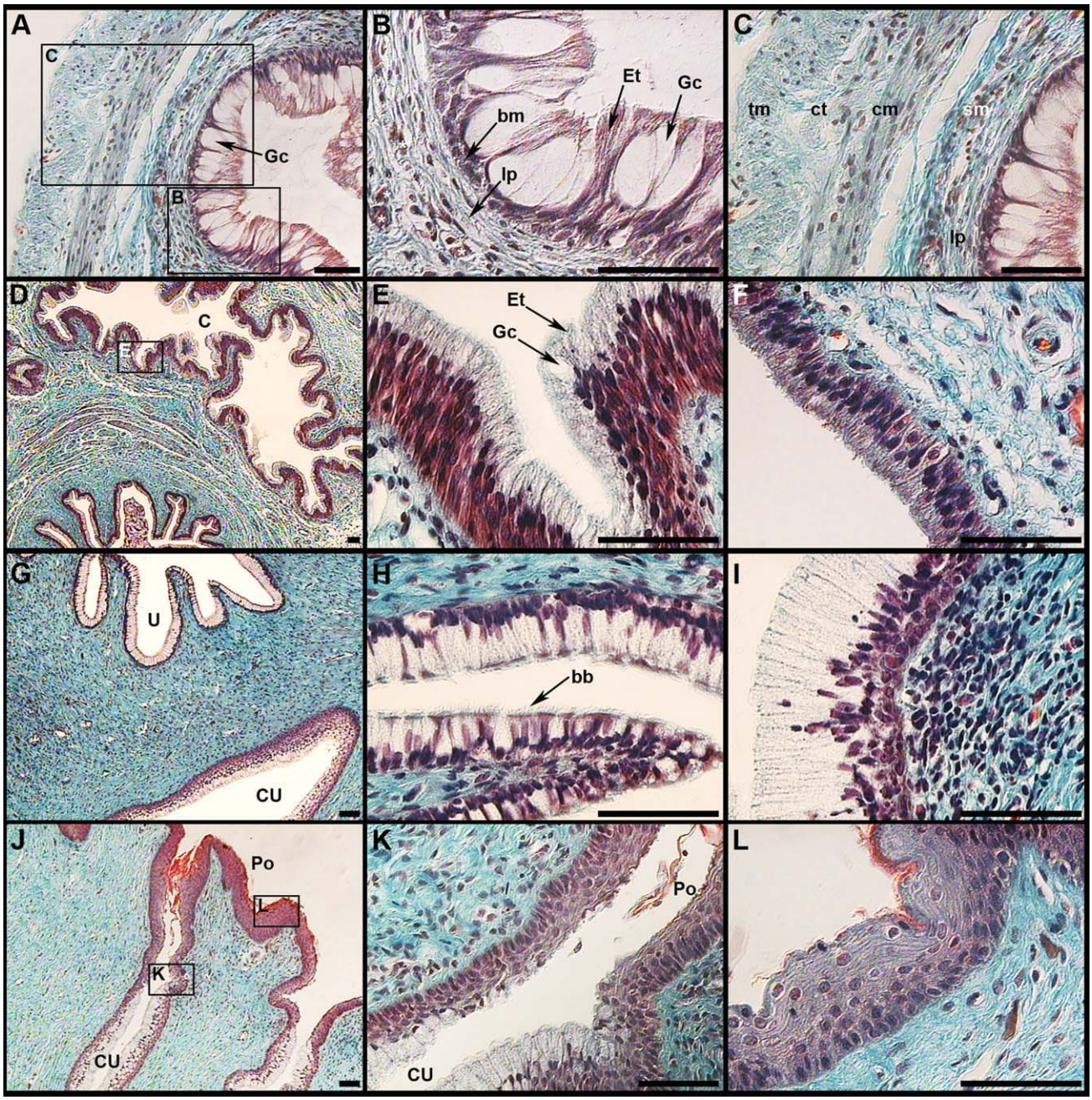

Fig. 2. Representative sections of colon (**A–C**), urodeal sphincter (**D–F**), urodeum (**G–I**), and proctodeum (**J–L**) of watersnakes. No differences in morphology were detected between species; sections shown are from *N. c. clarkii*. Areas of higher magnification (typically middle and right panels) are indicated by the boxed areas in low-magnification images (typically left panels). bm, basement membrane; bb, brush border; cm, circular muscle; CU, common urodeum; ct, connective tissue; Us, urodeal sphincter; Et, enterocyte; Gc, goblet cell; lp, lamina propria; U, L/R urodeal chambers; Po, proctodeum; sm, submucosa; tm, transverse muscle. Images produced using Masson Trichrome stain and differential interference microscopy. Scale bars = 50 μm.

the ampulla urogenital papilla (Fig. 3D). The ductus deferens is supported by a thin submucosa and a transverse muscle layer (Fig. 3E) and the epithelium is unlike that of any other urogenital organ in these species. The ductus deferens epithelium is columnar with a low pseudostratified/transitional layer (Fig. 3F). The nuclei are positioned basally and the cytoplasm stains darkly with Trichrome except at the apical-most tips of the cells (Fig. 3F). The apical membranes of these epithelial cells also appear to be convex, rather than flat as is seen in most other epithelia, and lack a brush border (arrowheads in Fig. 3F). In both sexes, the ureters communicate with the common urodeal chamber through a structure called the ampulla uriniferous papilla (females; *sensu* Siegel et al., 2011a) or

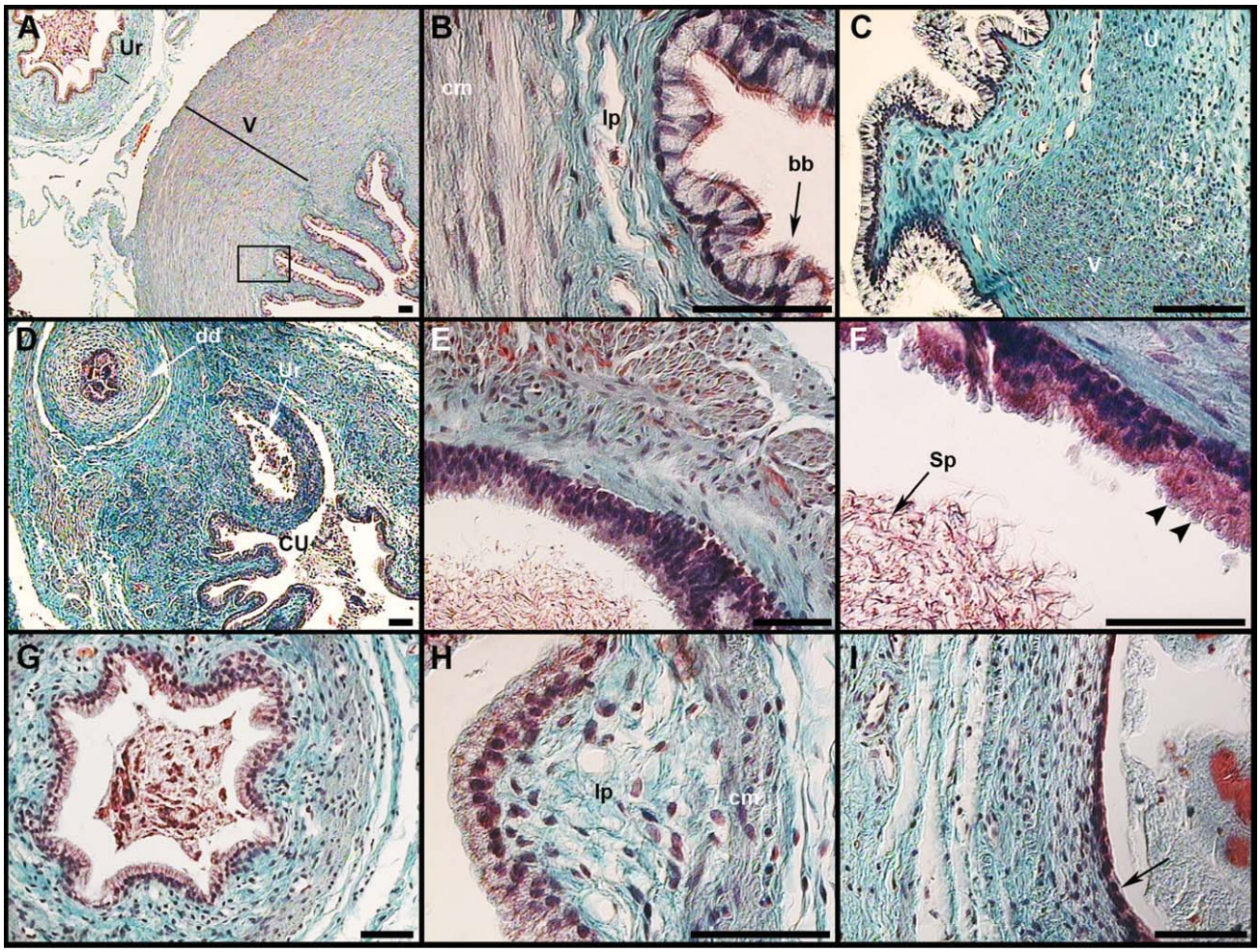

Fig. 3. Representative sections of the posterior vagina (**A–C**), ductus deferens (**D–F**), and ureters (**G–I**) of watersnakes (images shown are from *N. c. clarkii*). Arrowheads in F indicate convex apical membranes of epithelial cells in the ductus deferens. The arrow in I points to the low stratified squamous epithelium of the ureter where it meets the common urodeal chamber. bb, brush border; cm, circular muscle; CU, common urodeal chamber; dd, ductus deferens; lp, lamina propria; U, L/R urodeal chamber; Ur, ureter; V, vagina; Sp, sperm. Images produced using Masson Trichrome stain and differential interference microscopy. Scale bars = 50 μm.

ampulla urogenital papilla (males; Fig. 3D). The ureters are supported by a thick submucosa but only a very thin circular muscle layer (Fig. 3G); the urothelium (epithelium of the ureter) is simple cuboidal/short columnar with basally positioned nuclei, clear-staining apical cytoplasm (Fig. 3H), but becomes low squamous epithelium where the ureter transitions into the ampullae and joins the common urodeum (Fig. 3I). All subsequent analyses of ureter structure and function in both males and females were made cranial to the transition from the ureter proper to the ampullae.

Evidence for Mucus Secretion in the Colon/Cloaca

Using the Alcian blue (pH 2.5) and Periodic Acid Schiff staining techniques, we have detected acid and neutral mucins, respectively, in many of the intestinal/cloacal and reproductive epithelia. Both acid and neutral mucins were detected in the goblet cells (*) and the apical cytoplasm (arrowheads) of the colon (Fig. 4A,B) and urodeal sphincter (Fig. 4C,D) and in the apical cytoplasm of the urodeum (Fig. 4E,F). The proctodeal epithelium was negative for acid mucins (Fig. 4G) but positive in the region of the basement membrane for neutral mucins (Fig. 4H). Among the reproductive epithelia, the vaginas (Fig. 5A,B) stain positively throughout the epithelium for acid and neutral mucins, whereas only the apical margin of the cytoplasm from the ductus deferens was positive for mucins (Fig. 5E,F). The urothelium is positive for both acid and neutral mucins in both sexes (Fig. 5G,H; ureters pictured are from a male).

Distribution of NKA, NKCC, and AQP3/ Effects of Salinity

NKA was not detectable in the colon (Fig. 6A) or any of the cloacal chambers (Fig. 6D,G,J) of either

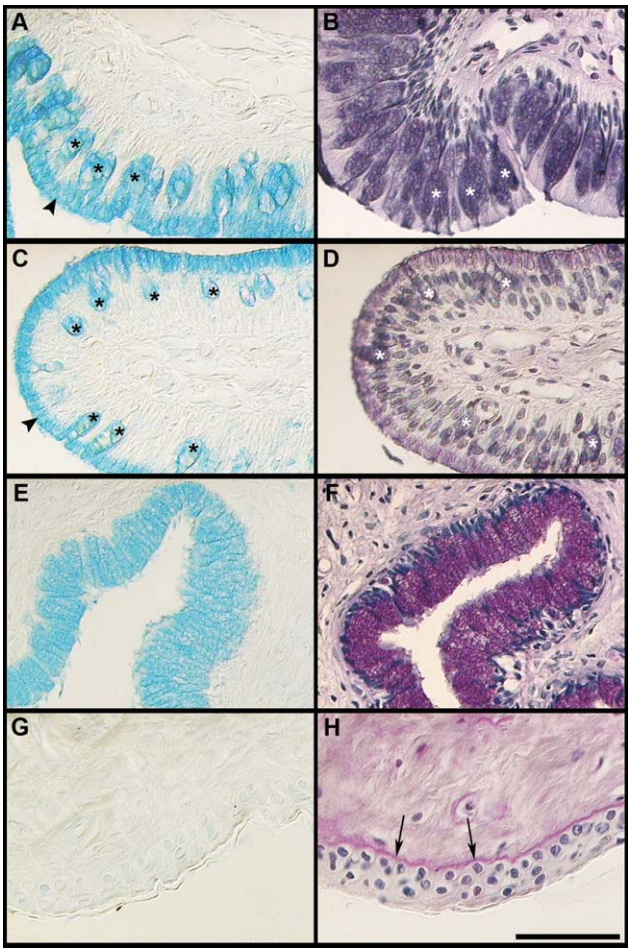

Fig. 4. Representative sections of epithelium from the colon (**A,B**), urodeal sphincter (**C,D**), urodeum (**E,F**), and proctodeum (**G,H**) in *N. c. clarkii* stained using Alcian blue (A,C,E,G) and PAS (B,D,F,H). Stars indicate the position of goblet cells and arrowheads point to apical mucin-rich cytoplasm. Arrows in H point to basement membrane, staining positively for PAS. Images produced via differential interference microscopy. Scale bars = 50 μm.

species. By contrast, NKCC was detected in the basal cytoplasm of the cells lining the colon (Fig. 6B), urodeal sphincter (Fig. 6E), and proctodeum (Fig. 6K) in both species. AQP3 was patchy in the epithelium of the colon of both species and appears to be associated with the mucus-secreting columnar cells, rather than the goblet cells, in this tissue (Fig. 6C). AQP3 was undetectable in the coprodeal complex (Fig. 6F), faint in the basal cytoplasm of the urodeum (Fig. 6I) and absent from the proctodeum (Fig. 6L) in both species. Negative control sections completely lacked positive staining in all tissues (insets in Figs. 6A,D,G,J). Interestingly, NKA was absent from the vaginas (Fig. 7A) and ducti deferentia (Fig. 7D) but was basolateral in the epithelium of the ureter (Fig. 7G) in both species. NKCC was also absent from the vaginas (Fig. 7B) in both species but was detected in the apical cytoplasm of the ducti deferentia (Fig. 7E). NKCC was absent from the urothelium (Fig. 7H). The epithelia of the vaginas (Fig. 7C) and the ducti deferentia (Fig. 7F) were negative for AQP3; however, the sperm in the duct pictured in Figure 7F were AQP3 positive. The ureters (analyzed cranially to the junction with the ampullae) of both species and both sexes stained positively for AQP3 in the basal cytoplasm (Fig. 7I). Again, all negative control sections lacked positive staining (insets in Figs. 7A,D,G). There was no effect of salinity on the general morphology of any of the colonic/cloacal epithelia, including the proportion of goblet cells in the colon (data not shown). There was also no effect of salinity on the distribution of mucopolysaccharides in the colonic/cloacal epithelia (data not shown), nor on the distribution of NKCC or AQP3 in the colon/cloaca (Fig. 8). Salinity acclimation also did not affect the distribution/localization of NKA or AQP3 in the urothelium (Fig. 9) of either species; however, the abundance of NKA appears to increase in 100% SW, relative to 0% SW, and the abundance of AQP3 appears to decrease in 100% SW, also relative to 0% SW.

DISCUSSION

The morphology and putative membrane transport function of the cloaca in aquatic snakes has

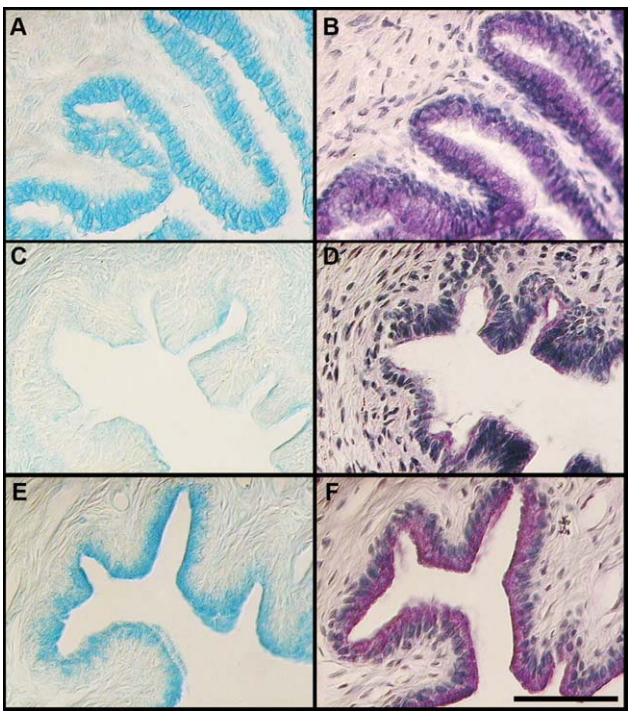

Fig. 5. Representative sections of epithelium from the vagina (**A,B**), ductus deferens (**C,D**), and ureters (**E,F**) in *N. c. clarkii* stained using Alcian blue (A,C,E) and PAS (B,D,F). Images produced via differential interference microscopy. Scale bars = 50 μm.

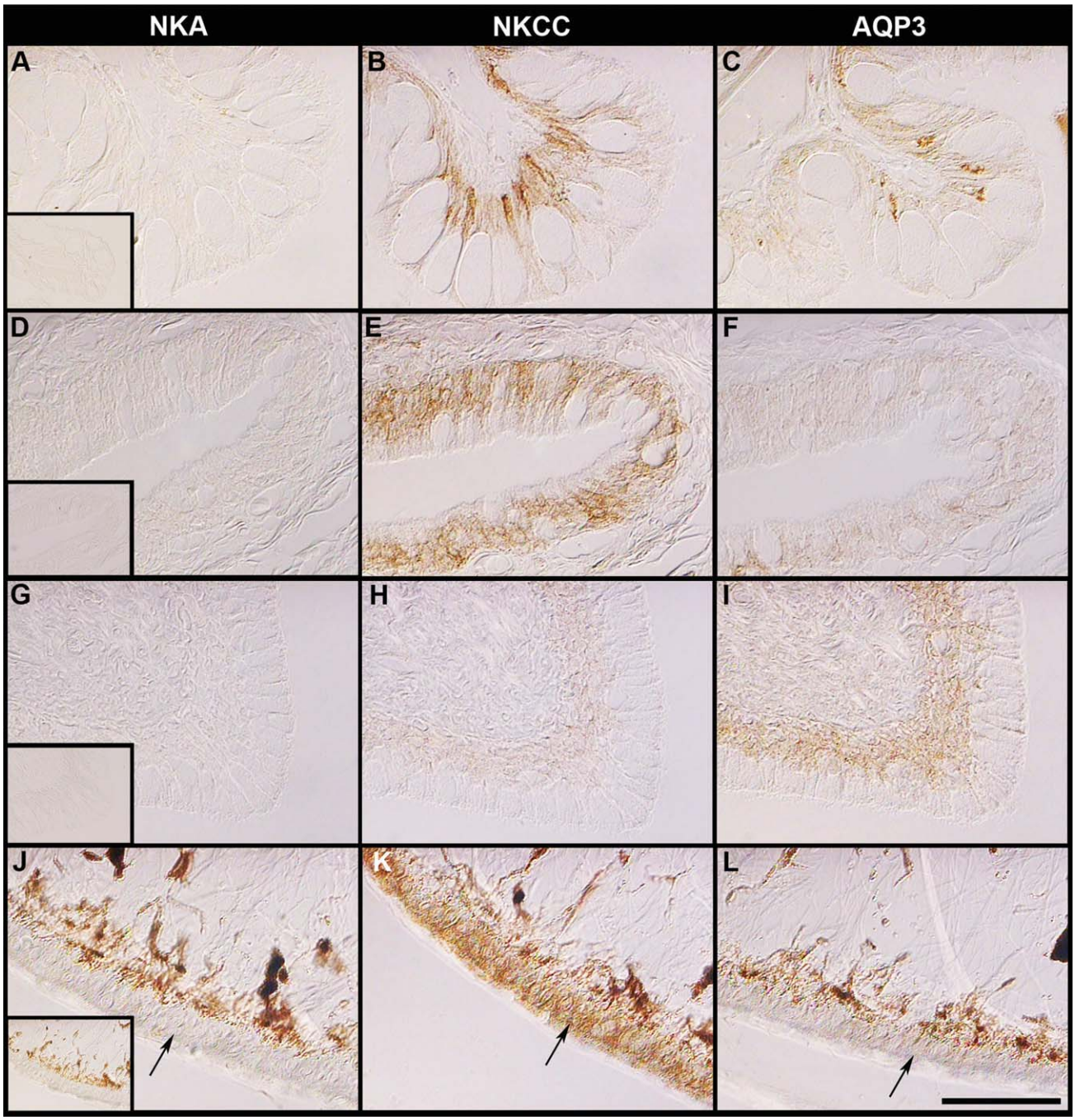

Fig. 6. Immunolocalization of NKA, NKCC, and AQP3 in the colon (**A–C**), urodeal sphincter (**D–F**), urodeum (**G–I**), and proctodeum (**J–L**) of watersnakes Sections shown are from *N. c. clarkii* in the control treatment (see Materials and Methods); no differences were seen between species under control conditions. The insets in panels A, D, G, and J show negative control sections for the colon, urodeal sphincter, urodeum, and proctodeum, respectively. Arrows in J, K, and L point to the proctodeal epithelium (positive for NKCC only). Images produced via differential interference microscopy. Scale bar = 50 μm.

never been examined, despite suggestions that the cloaca may contribute to urine modification in these animals (Yokota et al., 1985). Thus, the objectives of this work were to describe the morphology of the colon and cloaca in closely related species of snakes occupying different habitats (marine vs. freshwater) and to use knowledge of the relationship between form and function to hypothesize about the putative physiology of these organs in the context of epithelial ion and water transport. Because reabsorption of NaCl from the proximal tubule of the mammalian kidney is

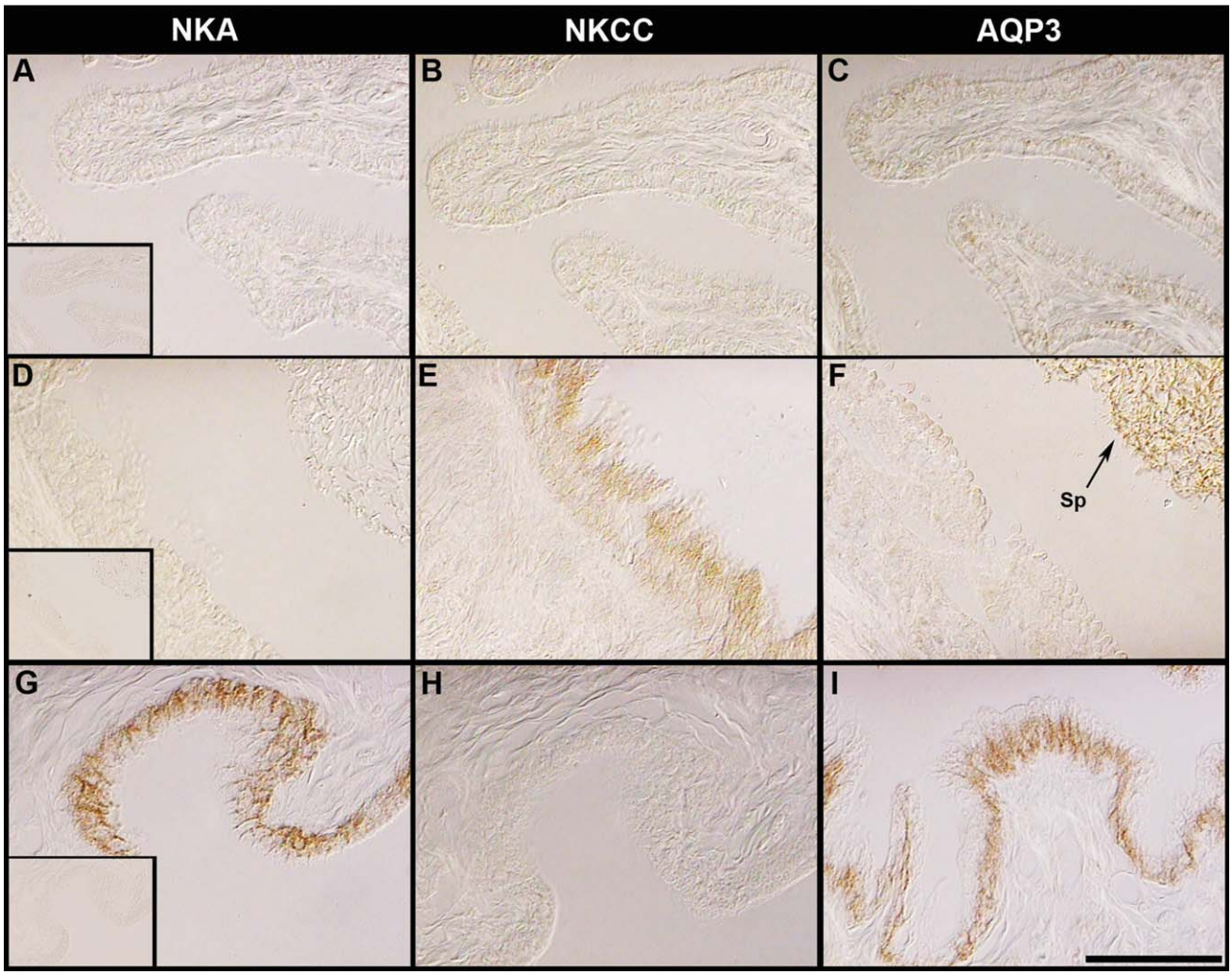

Fig. 7. Immunolocalization of NKA, NKCC, and AQP3 in the vagina (**A–C**), ductus deferens (**D–F**), and ureters (**G–I**) of watersnakes. Images shown are from *N. c. clarkii* under control conditions; no differences were seen between species under control conditions. Negative control sections for these tissues are shown as insets in panels A, D, and G, respectively. The arrow in F points to AQP3-positive sperm. Images produced via differential interference microscopy. Scale bar = 50 μm.

known to rely on the combined actions of an apical NaCl symporter (NKCC2) and basolateral NKA (Kinne and Zeidel, 2009) and because several reptilian taxa have been shown to reabsorb NaCl across the cloacal membranes (Schmidt-Nielsen and Skadhauge, 1967; Minnich, 1970; Skadhauge and Duvdevani, 1977), we expected to find apical NKCC and basolateral NKA in the urodeum and coprodeal complex. Surprisingly, these are not the results we observed. Additionally, while previous studies of membrane anatomy and physiology in the cloaca of terrestrial snakes suggested that both the colon and cloaca are important sites of Na^+ and water reabsorption (Seshadri, 1959; Junqueira et al., 1966), our results suggest that the colon, urodeal sphincter, and ureter might be important sites of ion transport, and, that the colon and common urodeal chamber may be important sites of solute-independent water reabsorption in aquatic snakes. Though we were unsuccessful in identifying differences between the marine and freshwater species, the results presented here suggest that further studies of the putative osmoregulatory function of the watersnake cloaca are needed.

Colon/Coprodeal Complex

The colon and the urodeal sphincter of both species of watersnake are typified by extensive infolding, suggesting both undergo expansion at times, possibly associated with the storage of feces and/or urine. While the colon is a simple columnar epithelium with abundant mucus-secreting goblet cells, the epithelium of the urodeal sphincter is pseudostratified and becomes more cuboidal than

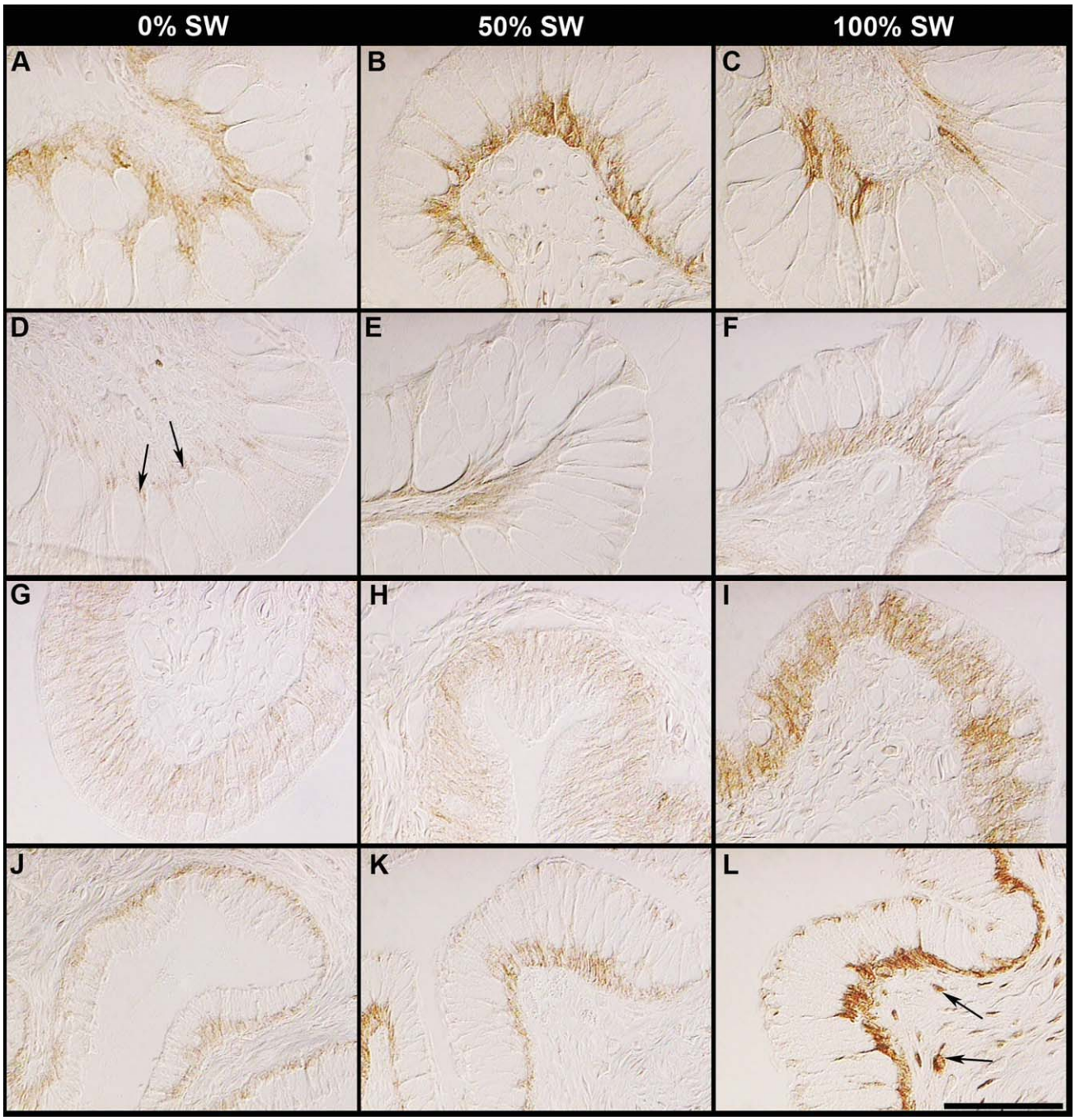

Fig. 8. Immunolocalization of NKA, NKCC, and AQP3 was not affected by treatment in either species; images shown are from *N. c. clarkii*. (**A–C**) NKCC is basal in the colonic epithelium across treatments. (**D–F**) AQP3 is basal (though nearly absent in the 0% SW treatment) in the colon of animals from all treatments. (**G–I**) NKCC is basal in the urodeal sphincter from all treatments, as is AQP3 in the (**J–L**) urodeal sphincter, and (**M–O**) urodeum. Treatments are indicated at the top of the figure. Images produced via differential interference microscopy. Scale bar = 50 μm.

columnar as it progresses toward the proctodeum (Fig. 2). This posterior transition from colon to the junction with the urodeum and, eventually, to the proctodeum, is also marked by a dramatic decrease in the proportion of goblet cells in the epithelium from $\gg$2/3 in the colonic epithelium to $\ll$1/3 in the urodeal sphincter to complete absence in the proctodeum (Fig. 4). These observations are similar to the results of previous studies of cloacal morphology in terrestrial snakes (Junqueira et al., 1966), crocodiles (Kuchel and Franklin, 2000), and birds (Johnson and Skadhauge, 1975), and suggest that

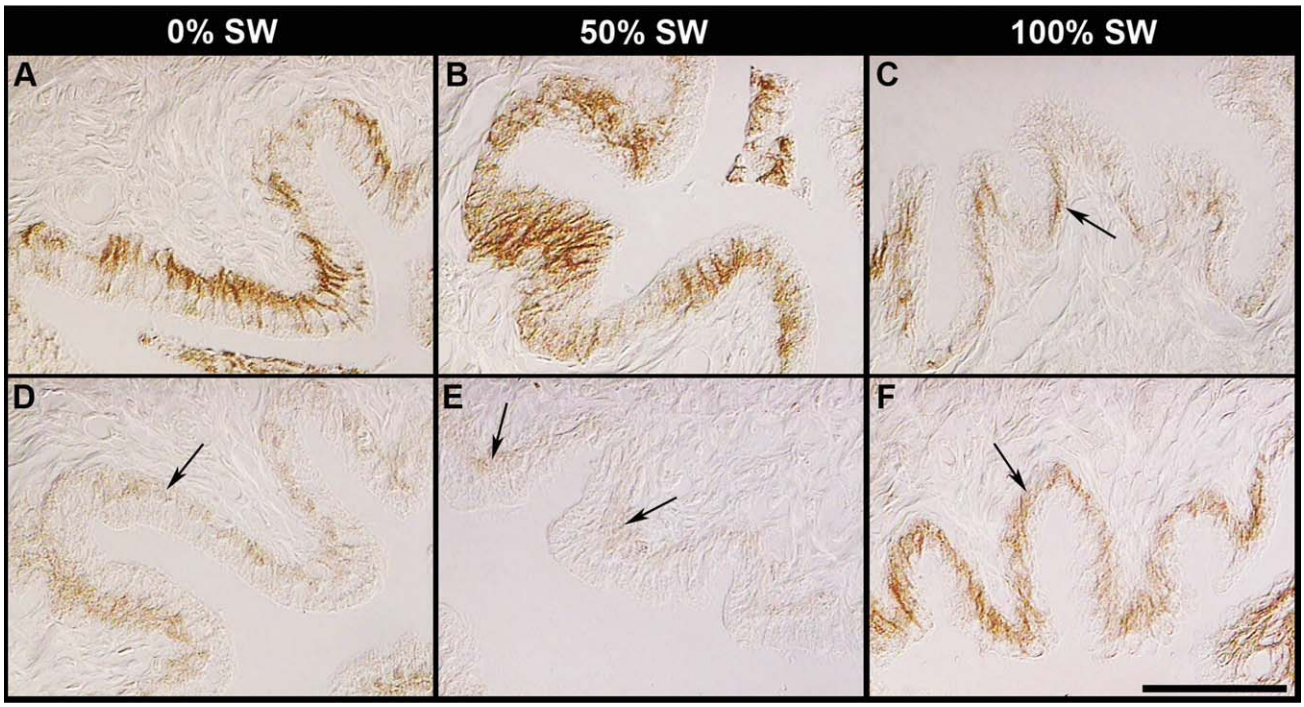

Fig. 9. Immunolocalization of (**A–C**) NKA and (**D–F**) AQP3 was not affected by treatment in the ureters, however, the abundance of NKA appears to decrease in 100% SW whereas AQP3 appears to increase in 100% SW. There was no difference between species; images shown are from *N. c. clarkii* and treatments are indicated at the top of the figure. Images produced via differential interference microscopy. Scale bar = 50 μm.(to Harvey B. Lillywhite) and the National Science Foundation (IOB-0519579 to DHE; EAPSI Fellowship to LSB).

the morphological transition from colon to proctodeum is associated with a transition in function from primarily secretion to primarily absorption. The presence of basolateral NKCC in the enterocytes of the urodeal sphincter (Fig. 6) support a potential role for this tissue in ion absorption, though the identity of the putative apical Na^+ channel/transporter remains to be determined. Interestingly, the epithelium of the colon is also positive, basolaterally, for AQP3 (Fig. 6). Whether these results suggest a role for the colon in fecal dehydration or if the localization of AQP3 merely reflects a role in the production of the watery component of mucus secreted by this tissue cannot be resolved at this time.

Urodeum and Proctodeum

Like the urodeum of crocodiles (Kuchel and Franklin, 2000) and birds (Johnson and Skadhauge, 1975) and the 'genital sinuses' of the snakes studied by Seshadri (1959), the L/R urodeal chambers in both species of watersnake are typified by tall columnar cells and long, thick rugae in both sexes (Fig. 2). Thick rugae are also present in the common urodeal chamber, suggesting that this portion of the cloaca undergoes dramatic expansion at times. The fact that these large rugae are present in both males and females suggest this expansion may be associated with the storage, and potentially the modification, of large amounts of urine (rather than reproductive function), as has been suggested among crocodiles (Kuchel and Franklin, 2000). Interestingly, both the L/R and common urodeal chambers stain positively for acid and neutral mucins (Fig. 4). Again, considering that this is true of both males and females, these data suggest that the presence of the apical mucin granules is associated with urinary function, rather than reproductive function. In particular, apical mucopolysaccharides in postrenal epithelia have been hypothesized to play a role in protection of the urinary system from blockage associated with precipitation of urate salts (More, 1977; Williams and Nicholson, 1983). While the urodeal mucins indentified in this study may, indeed, play a protective role in this tissue, the fact that we have not identified differences in the domain of expression of apical mucins between species or within a species between treatments suggests either that these two species do not differ in their response to salinity, or, that animals already have the appropriate amount of mucin in this tissue and further increases in the production of urinary products is not reflected in coincident increases in apical mucin. Further examination of the distribution of urinary products in the various segments of the cloaca during times of dehydration and saline load as well as confirmation of the putative protective function of the mucins will be necessary to

test this hypothesis. The proctodeum in both species was determined to be stratified squamous epithelium, as has been described among birds and several other species of snake (Johnson and Skadhauge, 1975; Siegel et al., 2011a, and references therein). The fact that the proctodeum is negative for both acid and neutral mucins (Fig. 4) and negative for AQP3 (Fig. 6) suggests this tissue is probably not involved in urine modification. The basal localization of NKCC in this epithelium (Fig. 6) may contradict this hypothesis, so future studies of ion transport in this tissue are of great importance. In particular, to support the hypothesis that the proctodeum plays a negligible role in water transport, it is necessary to demonstrate a lack of other potential AQPs in this tissue. Further, studies of putative ion transport across the basolateral membrane following inhibition of NKCC are also necessary to evaluate the contribution of this tissue to postrenal regulation of urine ion content.

Urogenital Ducts

Although the vagina in these species exhibits long thin rugae, similar to those of the L/R urodeal chambers, the epithelium of the vagina is surrounded by a very thick circular muscle layer, which is more basophilic than the circular muscle underlying the urodeum (Fig. 3). These results are similar to observations made on the uterus/vagina in other snake species (Uribe et al., 1998; Sever and Ryan, 1999). By contrast, the circular muscle layer surrounding the ureters is very thin, and this layer is absent from the supporting tissue around the ductus deferens (Fig. 3). These differences likely reflect the role of the muscular uterine wall in contracting during parturition and suggest that that similar contractions are not used in the expulsion of the urinary and seminal fluid from the ureters and ducti deferentia, respectively. The simple columnar epithelium of the vagina (Fig. 5A,B) secretes products comprised of both acidic and neutral mucins. By contrast, the epithelium of the ductus deferens, which was largely AB^- and PAS^-, appears to produce little to no mucin (Fig. 5C,D). Similar results were reported from examinations of the reproductive tract of female Mexican ground snakes, leading the authors to suggest that the vaginal secretions represent the female contribution to the production of a copulatory plug (Uribe et al., 1998). Copulatory plugs have not, to our knowledge, been identified in *N. c. clarkii* or *N. fasciata* but they are common in other Natricine snakes (Devine, 1975). This suggests that the mucus production in the vagina of female *N. c. clarkii* and *N. fasciata* may also contribute to the production of the copulatory plug, as hypothesized by Uribe et al. (1998). Further studies comparing the epithelial morphology and the production and/or secretion of AB^+/PAS^+ secretory material by the posterior vagina during the various stages of the reproductive cycle (especially during courtship) in these species would further aid in understanding the relative contributions of the male and female to the production of the copulatory plug, as well as other potential functions of mucus in this tissue. As is true of other reptiles (Liu, 1962), the ureters of both species of watersnake exhibit positive reaction for AB and PAS, which, like the presence of AB^+ and PAS^+ material in the urodeum, is consistent with a role for mucopolysaccharides in protection of the urinary system from blockage and or damage by the passage of urinary waste products. Interestingly, the presence of apical mucin has also been hypothesized to aid in the exchange of ions across the apical renal tubule epithelia (More, 1977 and references therein). While we find this hypothesis to be particularly interesting in the context of our goals in this study, we cannot evaluate the putative function of the apical mucins in the urothelium at this time. Because we failed to identify any differences in the presence or abundance of AB^+/PAS^+ material in any of the chambers of the cloaca in the two species examined herein, it is unlikely that differential production of mucus and its potential osmoregulatory function may underlie differences in habitat use between *N. c. clarkii* and *N. fasciata*.

Putative Cloacal Osmoregulatory Function

The lack of NKA in any of the colonic/cloacal epithelia was surprising since previous studies of aquatic snakes have identified basolateral NKA in the sublingual salt glands (Babonis et al., 2009) and distal tubules of the nephron (Babonis et al., in press) using immunohistochemistry. Importantly, the lack of NKA immunoreaction in the colon and cloaca may reflect the very low abundance of NKA in these tissues, rather than its absence. Physiological studies aimed at quantifying active Na^+ transport in these tissues combined with studies aimed at blocking NKA activity (e.g., using ouabain) would be informative in understanding the potential involvement of NKA in cloacal ion regulation. Additionally, because we were unsuccessful in localizing NKCC to the apical membranes of any cloacal compartment, the identity of the apical Na^+ transport mechanism remains elusive. Thus, further studies attempting to identify the mechanism of apical Na^+ transport in these tissues are warranted. It is possible that, like the epithelium of the bladder in mammals (Smith et al., 1998) and the hindgut of some lizards (Bentley and Bradshaw, 1972), apical Na^+ transport in the cloacal epithelia of watersnakes is effected by ENaC rather than NKCC. Alternatively, apical Na^+ transport might be mediated by

one of the Na^+/H^+ exchangers, which are known to play a in Na^+ transport in the kidney of garter snakes (Dantzler et al., 1991) and the gastrointestinal tract of mammals (Zachos et al., 2005).

Previous studies of reptile cloacal physiology have suggested that K^+ is secreted into the urine during storage in the cloaca (Skadhauge and Duvdevani, 1977; Lauren, 1985). While studies of exocrine glands suggest that a basolateral localization of NKCC is consistent with a role in K^+ secretion from these tissues (Haas and Forbush, 2000), studies aimed at examining the response of NKCC during times of high K^+ load are necessary to support this idea. Additionally, identification of an apical K^+ channel in these tissues would provide further support for this hypothesis.

AQP3 was detected in the basolateral cytoplasm and membranes of the colon and, weakly, in the urodeum, though no immunolocalization was detected in the goblet cells of these tissues (Fig. 6). These results are consistent with the localization of AQP3 in the distal colon of the rat (Frigeri et al., 1995) and amphibians (Mochida et al., 2008; Pandey et al., 2010) and suggest a role for AQP3 in fecal dehydration in watersnakes, as has been shown in these other taxa (Ishibashi et al., 1994; Frigeri et al., 1995). Although Taplin (1985) previously suggested that crocodiles may reabsorb water from the feces when experiencing desiccating conditions, we are the first to suggest that this process may involve AQP3 in reptiles. AQP3 was expressed only weakly in the urodeum (Fig. 6); despite this, we hypothesize its role in this tissue to be related to the reabsorption of water and glycoproteins from the urine. Minnich and Piehl (1972) suggested that reptilian sauropsids form urate spheres to prevent the buildup of urate crystals in the urinary tract. Interestingly, these authors were able to reconstitute the typical crystal structure of squamate urine from the urate spheres upon drying them. This suggests that these urate spheres are indeed modified after they leave the kidney and before they exit the body and we suggest that this process involves dehydration by AQP3 in the urodeum.

We demonstrate a basolateral localization of AQP3 in the urothelium of water snakes (Fig. 7), which is consistent with the localization of this protein in the urothelium of rats (Spector et al., 2002). Furthermore, rats exhibited increases in the abundance of AQP3 following dehydration (as demonstrated by changes in the intensity of immunohistochemical staining and western blot; Spector et al., 2002). While our studies did not assess dehydration directly, animals acclimated to 100% SW do appear to experience an increase in urothelial AQP3, relative to animals in the 0% SW treatment (Fig. 9). In combination with the observation that NKA was also basolateral in the urothelium of the watersnakes (Fig. 7) and that the abundance of NKA appears to decrease with acclimation to 100% SW (Fig. 9), we suggest that the ureters of watersnakes, like those of some mammals (Spector et al., 2002, and references therein) may be involved in postrenal modification of the urine. Interestingly, the ureter of lizards has been shown to be inactive, with respect to urine modification (Roberts and Schmidt-Nielsen, 1966), making our results somewhat surprising. While true quantitative changes in protein abundance are necessary to support the hypotheses proposed herein, other studies of water regulatory proteins have, similarly, inferred changes in protein abundance from changes in the intensity of immunohistochemical staining (Ramirez-Lorca et al., 2006; Mochida et al., 2008). These putative changes in protein abundance in the urothelium suggest that watersnake ureters may exhibit physiological plasticity associated with habitat use; however, future studies quantifying changes in AQP3/NKA protein and mRNA abundance (using western/dot blots and quantitative real-time PCR, respectively) are necessary to fully understand the relationship between habitat salinity and ureteral ion/water transport in watersnakes. Additionally, considering that arginine vasotocin is known to increase Na^+ reabsorption (and also, likely, increase cloacal water permeability) in *Varanus gouldii* (Braysher and Green, 1970), and that the expression of AQP3 is known to be regulated by arginine vasopressin in mammals (Terris et al., 1996), it would be interesting to examine changes in the water and ion transport and the distribution and abundance of AQP3 in the watersnake cloaca in response to treatment with AVT. Finally, future studies aimed at analyzing the terminal segment of the urogenital ducts (the ampullae uriniferous/urogenital papillae), would be particularly useful in evaluating the potential for this structure to play a role in urine storage and/or modification (as originally hypothesized by Siegel et al., 2011b).

CONCLUSIONS

We provide initial analysis of the morphology of the colon, cloaca, and posterior reproductive structures of two species of watersnakes, one from a marine habitat and one from a freshwater habitat. The general lack of differences in the morphology/putative function of these epithelia suggests that the proteins we examined in this study may not contribute much to the ability of *N. c. clarkii* to survive in marine habitats. Further studies aimed at examining the transport properties of the cloaca of sea snakes, which have a salt gland, would make an interesting comparison with the results presented here and with the results of similar studies from other vertebrates that also have a salt gland.

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