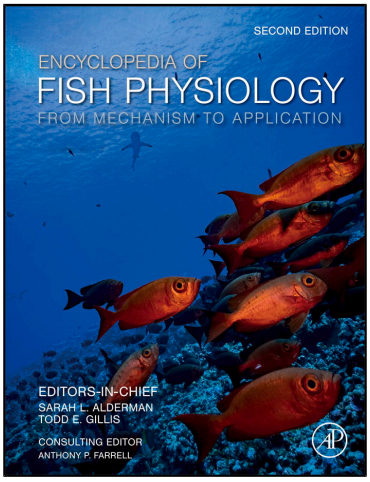

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Osmoregulation in chondrichthyan fishes

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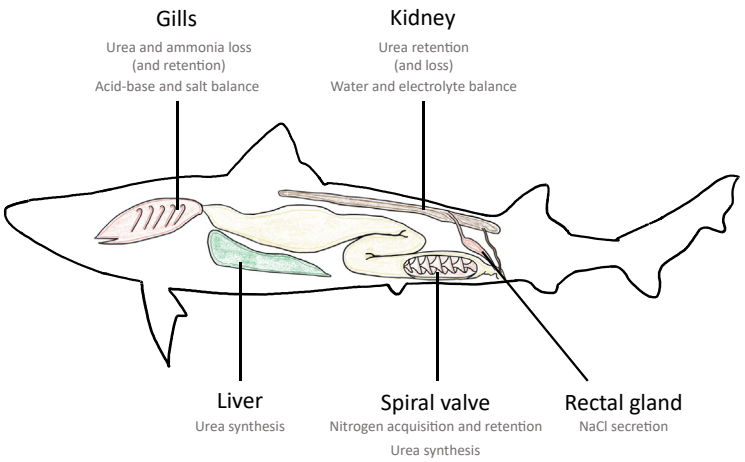
Key points

- Marine chondrichthyan fishes have adopted a ureosmotic strategy regulating ion balance and osmoconforming
- Major modifications in kidney and gill morphology aid in retention of urea in the body fluids. Inclusion of a rectal gland aids in balancing intermittent salt loads associated with feeding and/or drinking
- Amphihaline elasmobranchs such as the bull shark are also ureosmotic when in SW but retain significant amounts of urea in freshwater, whereas obligate freshwater elasmobranchs have abandoned urea as an osmolyte and function much like a freshwater teleost.

Abstract

Effective regulation of water and solute concentration is a life-sustaining physiological process in all organisms. Aquatic organisms use very different physiological strategies to deal with the solute-poor environments of freshwater versus the solute-rich environments of seawater. Chondrichthyan or cartilaginous fishes (elasmobranchs and holocephalans) have evolved a ureosmotic strategy where high concentrations of urea are retained such that the fish osmoconforms to the marine environment but notably, ions are regulated. Here we discuss the functional role of the major organs involved in this process, how their actions may be integrated, and how the physiology differs in a freshwater environment.

Teaching slide



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Introduction

All living vertebrates aim to maintain their internal environment at a stable state for optimal cell function in the face of a changeable external environment. In aquatic organisms, a major physiological challenge is the internal regulation of salt and water balance, particularly if the animal occupies different environmental salinities throughout its natural life cycle. Different strategies have evolved such that aquatic organisms osmoregulate or osmoconform depending on their external environment. Osmoregulators maintain the osmolality of their internal fluid environment within a reasonably narrow range regardless of the osmolality of the external environment. In fishes this strategy is most prevalent in actinopterygian fishes, where plasma sodium (Na^+) and chloride (Cl^-) concentrations and the resulting internal fluid osmolality are regulated at levels approximately one-third of seawater (SW). Importantly, in marine teleosts the maintenance of an internal environment that is hyposmotic to the external environment brings a serious dehydrating challenge with the constant movement of water out of the animal into the hyperosmotic environment. To cope with this water loss, marine teleosts drink the external SW environment, retain the water, and actively excrete excess ions from branchial ionocytes and the kidneys.

Conversely, the majority of marine invertebrates and hagfishes are osmoconformers, where internal body fluid osmolality and ion concentrations are maintained at similar concentrations to the marine environment. This is a risky strategy if environmental salinity is variable, as the animals are unable to combat the movement of water, and therefore cannot regulate body fluid volume very effectively. Thus, osmoconforming animals typically inhabit stenohaline environments where there is little to no change in the osmolality of the external environment.

A third strategy has evolved in the chondrichthyan fishes (elasmobranchs and holocephalans), where the primary ions, such as Na^+ and Cl^-, are regulated but the internal body fluids of the fish osmoconform to the external marine environment. This strategy was first reviewed by Homer Smith in his seminal series of publications on osmoregulation in fishes (Smith, 1931). To maintain this strategy, marine chondrichthyans regulate plasma ion concentrations to levels approximately half that of the surrounding SW, but at the same time retain high concentrations of osmotically active nitrogenous compounds, such as urea and methylamine. This results in a ureosmotic strategy where the concentration of urea is maintained at 350–450 mM in the extracellular fluid, accounting for approximately one-third to one-half of the total osmolality, and the retained methylamine acts to buffer enzyme function against the perturbing effect of increasing urea concentrations. Concentrations of both urea and methylamines have also been shown to aid in buoyancy in elasmobranch fishes, and there is a known effect of depth on circulating levels of these osmolytes, with deep sea dwelling elasmobranchs retaining more methylamines and less urea (Yancey, 2016). Regardless, the osmolality of the body fluid of chondrichthyan fishes is similar to, or slightly hyperosmotic, to the surrounding SW, thereby limiting water movement out of the animal as well as the risk of dehydration in a marine environment.

Euryhaline elasmobranchs

Euryhalinity implies that the organism can adapt to, and survive in, a wide range of salinities; however, there are degrees to which this range extends. Amphihaline fishes are the champions of a euryhaline approach and will migrate between full strength SW and freshwater (FW) environments as part of their natural life-cycle (Zydlewski and Wilkie, 2013). Teleosts such as salmonids and eels have been widely used for research on body-fluid regulation, as they switch between a hyperosmoregulating animal to a hyposmoregulating animal during their migration between FW and SW environments. These fishes have provided immense insight into our understanding of how iono- and osmoregulating mechanisms are controlled at the gills, intestine, and kidneys to effectively maintain salt and water balance across these extreme environments.

There are approximately 1250 known species of chondrichthyan fishes with greater than 90% considered obligate marine species that adopt a ureosmotic strategy. However, approximately 43 species of shark, skate, and ray from 4 families and 10 genera are known to have some described level of euryhalinity, and will acclimate to varying limits of salinity as part of their natural life cycle (Martin, 2005). Known amphihaline species include the bull shark *Carcharhinus leucas*, Atlantic stingray *Hypanus sabinus* and largetooth sawfish *Pristis microdon*. The bull shark is found in tropical and subtropical regions circumglobally. As a viviparous species, female bull sharks give birth to live young in estuarine areas and new-born pups are known to immediately migrate upstream into a fully FW environment, occupying inshore rivers as nursery habitats. The reasons for these behaviors are unclear but likely reflect the available resources in highly productive FW systems in tropical regions, alongside avoidance of predation from marine predators such as other shark species.

In a SW environment, bull sharks and other euryhaline elasmobranchs conduct a ureosmotic strategy much in the same way as obligate marine elasmobranchs. However, in a FW environment euryhaline elasmobranchs retain high levels of Na^+, Cl^- and urea, even though they appear to lose a greater proportion of urea from their extracellular fluid, such that their plasma osmolality approximates to 600 mOsm, almost twice that of FW adapted teleosts. This presents an enormous challenge in regulating body fluid volume, as the constant inwardly directed osmotic gradient encourages movement of water into the animal. Thus, much like salmon and eels, in FW the amphihaline elasmobranch excretes excess water by renal routes but retains osmolytes by renal, branchial, and other routes. Unlike salmon and eels, our understanding of the physiological mechanisms of branchial, renal, and intestinal function of amphihaline elasmobranchs in a FW or SW environment are less clear, with perhaps the exception of the rectal gland, a specialized salt secreting gland found in both elasmobranchs and holocephalans that secretes a solution isosmotic to SW that is almost entirely composed of NaCl (see below).

In the following sections, we summarize the role of key organs responsible for maintaining whole body solute and water balance in chondrichthyan fishes. Further, we provide some insight into the physiological mechanisms that enable this strategy and how these mechanisms may be integrated through the actions of vertebrate hormone systems that are known to exist in chondrichthyan fishes, but our understanding of their function is weak (Hara et al., 2018). Much of our text is by necessity focused on elasmobranchs, as much less is known about the holocephalans, which we will reference when applicable.

Liver

As in all vertebrates, the elasmobranch liver acts as an energy source to provide fuel for the function of extrahepatic tissues. Elasmobranchs are different from bony fishes as they appear unable to use fatty acid metabolism as a major fuel source. Rather, they will utilize both carbohydrates (glucose) and ketones (β-hydroxybutyrate) as the major fuel sources, and evidence suggests some tissues rely on ketones for optimal function. Indeed, both gluconeogenesis and ketogenesis have been documented using isolated hepatocytes (Mommsen and Moon, 1987). Furthermore, the elasmobranch liver contains as much as 52−83% lipid by mass in *Squalidae* species (Wetherbee and Nichols, 2000) and plays a critical role in buoyancy due to their lack of a swim bladder.

In the context of osmoregulation, the liver has received some attention given it is a primary site of synthesis for urea and methylamines. The hepatic ornithine urea cycle (OUC) is the biochemical pathway responsible for the detoxification of ammonia from protein catabolism and the synthesis of urea in all vertebrates. However, in elasmobranchs glutamine is the primary nitrogen donor as opposed to free ammonia in other vertebrates (Anderson, 1991). There is a growing body of evidence indicating significant urea synthesis in extrahepatic organs, such as muscle, intestine, and kidney (Kajimura et al., 2006; Takagi et al., 2012), while the precise role of this remains unclear, it undoubtedly contributes to the overall urea balance in elasmobranchs. Most studies have shown when partially euryhaline elasmobranchs, such as the little skate, *Leucoraja erinacea*, European catshark, *Scyliorhinus canicula*, or Pacific spiny dogfish, *Squalus suckleyi* are acclimating to reduced salinity, they will dump urea to reduce their plasma osmolality to match the environment. Conversely, when acclimating to increased salinity, urea is retained. Our understanding of methylamine regulation in elasmobranchs is weak but similar relationships between salinity and plasma methylamine concentration have sometimes, but not always, been demonstrated. Regulation of urea balance and salinity is likely achieved through a combination of hepatic urea production and renal urea loss. Reduction in urea loss as the animal acclimates to increased salinity may be a function of reduced urine flow rate (UFR) alongside enhanced reabsorption by renal tubular mechanisms (see below). A limited number of studies have examined direct effects of salinity adaptation on hepatic urea synthesis, and have indicated a positive correlation between urea production and/or hepatic OUC enzyme activity and salinity (Anderson et al., 2005). Hormonal control of hepatic urea production in elasmobranchs has not been investigated, but some studies have investigated hormonal regulation of metabolic processes in elasmobranchs which may be linked to urea production through the catabolism of amino acids as a metabolic fuel, yielding ammonia—a substrate for urea synthesis (Speers-Roesch and Treberg, 2010).

The exception to the rule, in regard to urea balance in elasmobranch fishes, are the *Potamotrygonidae* rays. These are obligate FW rays of the Amazon that have lost the ability to synthesize significant amounts of urea in their liver. Indeed, it seems that this group of elasmobranchs have abandoned a ureosmotic strategy and adopted an osmoregulatory strategy more akin to that of FW teleosts.

Kidney

Osmoregulating marine vertebrates constantly deal with the risk of dehydration, and thus excrete only small amounts of urine to avoid excessive water loss. However, in the slightly hyperosmotic marine elasmobranchs a net influx of water leads to typically higher urine flow rates compared to marine teleosts. This is advantageous for maintenance of internal homeostasis because fish can excrete metabolic waste via the urine. However, it also comes at a cost to the ureosmotic marine elasmobranch as the requirement to retain urea by the kidneys to maintain the iso- or slightly hyperosmoregulatory strategy means much of the filtered urea must be reabsorbed.

During the course of urine production, urea in the plasma is freely filtrated by the glomerulus, but in the chondrichthyan fish kidney more than 90% of filtered urea is reabsorbed in the nephron to minimize urea loss (Kempton, 1953). Indeed, loss of urea by renal routes may account for 4−20% of total urea loss by the animal (Goldstein and Forster, 1971), with the extent of urea loss largely depending on the environmental salinity. Acclimation studies have demonstrated an increase in key renal parameters, such as UFR and glomerular filtration rate (GFR), as elasmobranchs are transferred to reduced environmental salinities. This reflects a need to remove excess water as the animal adapts to a hyposmotic environment but also results in the negative consequence of an increase in renal urea loss, resulting in a reduction of plasma urea (Wells et al., 2002). The ability of elasmobranchs to adapt to these changing salinities further reflects the importance of renal urea reabsorption. How urea reabsorption is achieved has not been completely resolved; however, it is evident that the renal tubules of marine cartilaginous fish are among the most elaborate in the vertebrate phyla, with unique features that have likely evolved for the specific purpose of retaining filtered urea (Lacy and Reale, 1985; Hyodo et al., 2014).

The chondrichthyan fish kidney consists of multiple lobules, with each lobule composed of two zones: a sinus or mesial zone, and a bundle or lateral zone. Beginning at the renal corpuscle, each nephron makes four turns going back and forth repeatedly between the two zones. The first and third loops are housed in the bundle zone, and the second and fourth loops are housed in

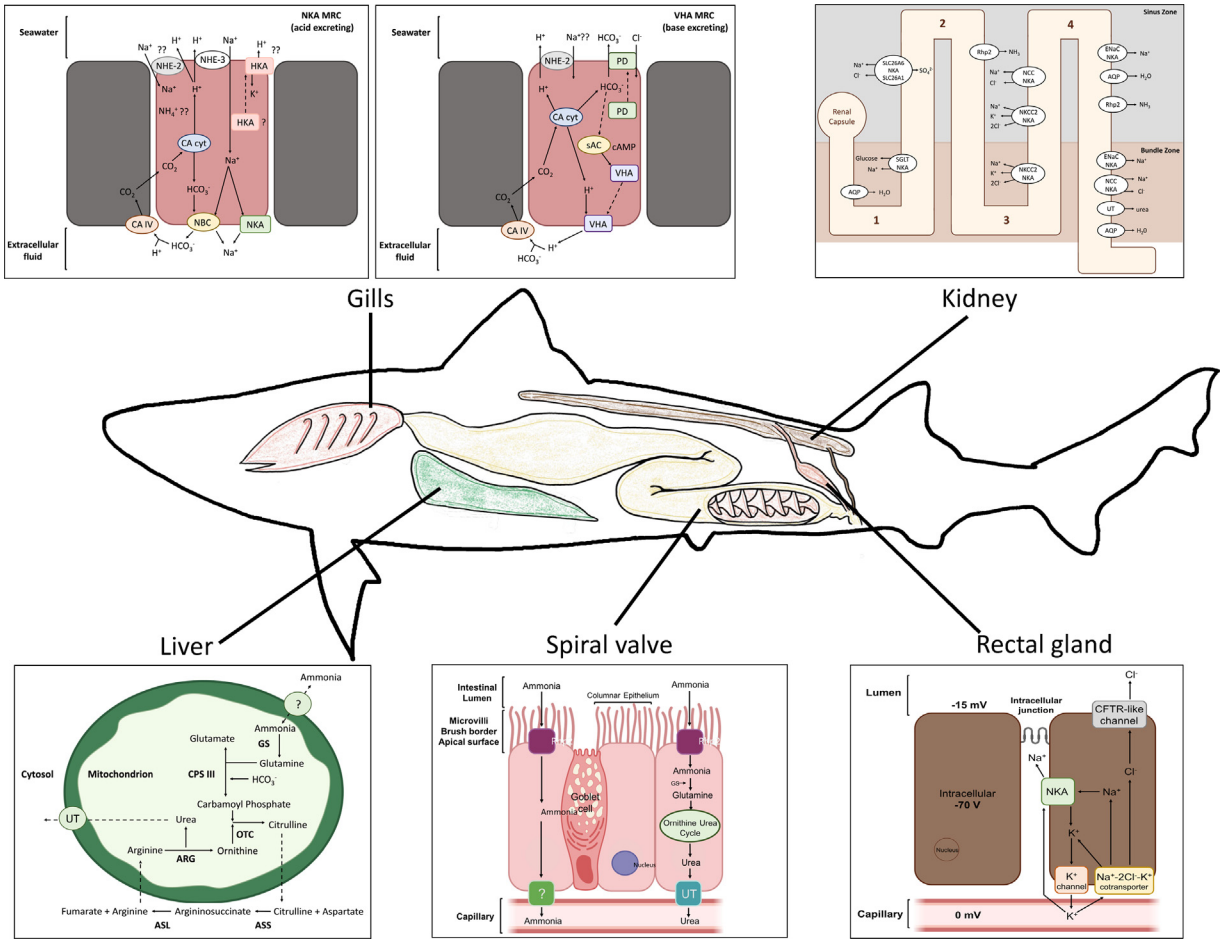

Fig. 1 Proposed models for acid-base regulation and NaCl uptake at the gills, urea reabsorption and water and salt regulation at the kidney, urea synthesis in the liver by the ornithine urea cycle, nitrogen handling in the gut, and salt excretion in the rectal gland of the marine adapted elasmobranch (See text for further details). GILL acid secreting ionocyte: NKA—Na^+/K^+-ATPase; NBC—Sodium bicarbonate co-transporter; CA IV—extracellular membrane associated Carbonic anhydrase; CA cyt—Cytosolic carbonic anhydrase; NHE-2 and NHE-3—sodium hydrogen exchangers; HKA—H^+/K^+-ATPase. GILL base excreting ionocyte: sAC—soluble adenylyl cyclase; VHA—V-type ATPase; PD—pendrin-like chloride/bicarbonate exchanger. Dashed lines indicate movement of proteins from the intracellular organelles to the membrane, solid lines indicate directional movement of solutes. KIDNEY proteins include NKA—Na^+/K^+-ATPase; SGLT—sodium glucose co-transporter; Slc26a1—sulfate transporter; Slc26a6—chloride/sulfate exchanger; AQP—aquaporin; NKCC—Na^+-K^+-$2Cl^-$cotransporter; NCC—Na^+-Cl^- cotransporter; ENaC—epithelial sodium channel; Rhp2—rhesus-like protein 2; UT—urea transporter. Ornithine urea cycle in mitochondrion of the LIVER: ARG—arginase; OTC—ornithine transcarbamoylase; CPS III—carbamoyl phosphate synthetase III; GS—glutamine synthetase; ASL—argininosuccinate lyase; ASS—argininosuccinate synthase; UT—urea transporter; ? - unknown. Putative transport of ammonia and urea across INTESTINAL epithelial cells: Rhp2—rhesus-like protein 2; GS—glutamine synthetase; UT—urea transporter; ? - unknown. Model for sodium and chloride secretion in the RECTAL GLAND: NKCC1—Na^+-K^+-$2Cl^-$cotransporter-1; NKA—Na^+/K^+-ATPase; CFTR—cystic fibrosis transmembrane regulator. GILL—Adapted from Tresguerres et al. (2005), Wood and Wright (2016), KIDNEY—Adapted from Hyodo et al. (2014), LIVER—Adapted from Anderson (1991), Wood and Wright (2016), INTESTINE—Adapted from Hoogenboom et al. (2023), RECTAL GLAND—Adapted from Silva et al. (1977).

the sinus zone (Fig. 1). The five tubular segments in the bundle zone are enclosed by a sac-like peritubular sheath, where cells are joined by tight junctions to form a boundary membrane that separates the microenvironment inside the sheath from the outside (Lacy and Reale, 1985). The unique features of the nephron segments imply that the bundle zone houses important machinery to maintain proper kidney function in chondrichthyan fishes (i.e., urea reabsorption). Indeed, in his initial studies of elasmobranch renal function, Boylan (1972) proposed that passive reabsorption of urea may be possible, provided a concentration gradient existed in a specific nephron segment. A facilitative urea transporter was first identified from the rat kidney. In mammals, two genes encoding UT-A (*slc14a2*) and UT-B (*slc14a1*) are tandemly located on chromosome 18. Transcripts of the UT-A gene are expressed in the inner medullary collecting duct (IMCD) and the thin descending limb of the loop of Henle. In the IMCD, urea is reabsorbed by UT-A proteins to create a urea-rich environment in the interstitium, which is essential for the urinary concentration mechanism in the mammalian kidney. The importance of UTs for urea reabsorption in mammals lead to their subsequent examination in chondrichthyans, and have been identified in numerous tissues in at least five species, with the highest mRNA abundance always

demonstrated in the kidney. In elephant fish, *Callorhinchus milii*, three UT genes (*ut-1*, *ut-2*, and *ut-3*) have been identified, with UT-1 playing a major role in renal urea reabsorption. In the houndshark kidney, UT is almost exclusively located in a single nephron segment in the bundle zone, the final segment of nephron (the collecting tubule) (Hyodo et al., 2004) (**Fig. 1**). The exclusive localization of UT in the collecting tubule has also been confirmed in elephant fish, bull shark, and red stingray *Hemitrygon akajei*. Following identification of the UT localization, molecular analyses have facilitated the mapping of membrane solute transporters and have revealed that the mechanisms important for reabsorption of NaCl, water, glucose, and urea are localized in the nephron segments inside the peritubular sheath (**Fig. 1**). Furthermore, based on these molecular mapping results, a hypothetical model for urea reabsorption has been proposed in the bundle zone, in which the unique configuration and permeability of nephron segments were accounted for. This model consists of four steps (Hyodo et al., 2014):

(1) Energy-dependent NaCl reabsorption driven by Na^+/K^+-ATPase and a Na^+-K^+-$2Cl^-$ cotransporter in the third loop of the nephron
(2) Passive water reabsorption possibly from the first loop of the nephron driven by the increased osmolality in the interstitial fluid by step 1
(3) Facilitative urea reabsorption occurs via specific urea transport proteins in the final segment of the nephron, and is driven by a large concentration gradient between the filtered urine (high urea) and the interstitial fluid (low urea) generated by steps 1 and 2
(4) Unidirectional flow of the reabsorbed interstitial fluid from inside the bundle to the blood sinuses via the single lymph capillary-like central vessel (Hentschel et al., 1998).

Messenger RNA (mRNA) abundance of solute transporters were upregulated in the kidney following transfer of the amphihaline bull shark (Imaseki et al., 2019) and red stingray (Aburatani et al., 2022) from hyperosmotic SW to hyposmotic FW environments. This suggests the resulting increase in GFR would lead to greater urea filtration into the nephron and would be compensated for by an increased renal reabsorptive capacity of NaCl and urea in the FW environment.

In addition to the importance of renal urea retention, the kidneys are also involved in regulation of plasma divalent ions. For example, magnesium (Mg^{2+}) and sulfate (SO_4^{2-}) are considerably higher in SW compared to the plasma of chondrichthyan fishes. To avoid hypermagnesemia and hypersulfatemia, chondrichthyan fish can concentrate divalent ions in the urine (26–182 mM) between 10 and 100 times higher than that in the plasma (0.5–8 mM). In the holocephalan elephant fish, a bladder-like structure has been described during the embryonic life stage; fluid within this structure contained Mg^{2+} (296.4 mM) and SO_4^{2-} (310.3 mM) at 300 times the concentration of the plasma (Hasegawa et al., 2016). The exact mechanisms of how this is achieved is unknown; however, sulfate transporters, Slc26a1 and Slc26a6, have been identified in the kidney of elephant fish, and functional expression analyses using *Xenopus* oocytes revealed that the elephant fish Slc26a1 was an electroneutral SO_4^{2-} transporter, while the elephant fish Slc26a6 was an electrogenic Cl^-/SO_4^{2-} exchanger (Hasegawa et al., 2016). The Slc26a1 and Slc26a6 proteins were co-localized in the basolateral and the apical membranes, respectively, of the proximal II segment (the second loop of nephron in the sinus zone), indicating that the second loop of the nephron may be the site for the secretion of excess divalent ions (Hasegawa et al., 2016). Indeed, previous micro-puncture studies suggested the existence of a secretory segment for divalent ions in the second loop of the little skate nephron (Stolte et al., 1977). In support of the findings in the elephant fish, mRNA abundance of *slc26a1* was decreased following transfer of the bull shark from the SO_4^{2-} rich SW habitat to the SO_4^{2-} poor FW habitat indicating the importance of Slc26a1 for SO_4^{2-} excretion via the kidney in a SW environment.

Hormonal control of renal function in vertebrates typically involves control of blood flow to the filtering glomerulus and/or modification of epithelial transport mechanisms. Studies examining hormonal control of elasmobranch renal function are few, with vasopressor hormones such as angiotensin II (Ang II) and arginine vasotocin (AVT) both inducing a predicted anti-diuretic response. However, evidence to date suggests that the action of these hormones is entirely vascular in nature, as perfusion of either AVT or Ang II induced a decrease in the population of filtering nephrons but had no effect on the relative clearance of Na^+, Cl^-, or urea. Conversely, the elasmobranch natriuretic peptide CNP was shown to induce both a natriuresis and diuresis as one might anticipate, but again, the actions seem confined to the vasculature (Wells et al., 2006).

Rectal gland

Elasmobranchs possess a specialized organ known as the rectal gland. This small digitiform gland is supplied by a single rectal gland artery that branches from the dorsal aorta, and splits into circumferential arteries to envelop the gland. In all elasmobranchs examined to date, the blood perfuses the secretory epithelia that line the secretory ducts and converge on one central vessel that empties into the colon. In holocephalans, the rectal gland is not a discrete structure but rather a series of tubular structures embedded within the wall of the post-valvular intestine (Hyodo et al., 2007). The rectal gland secretes a fluid that is typically iso-osmotic to the plasma of chondrichthyan fishes but is almost entirely composed of sodium chloride (Burger and Hess, 1960). A significant amount of research has been devoted to this organ, given the immense concentration gradients it operates against to actively excrete excess sodium chloride and retain urea. In essence, salt excretion occurs in the rectal gland epithelial cells through secondary active transport, where a basolaterally located Na^+/K^+-ATPase drives the inwardly directed movement of Na^+ and Cl^- via a basolateral positioned Na^+-K^+-$2Cl^-$ co-transporter. Cl^- then leaves the cell via an apically located CFTR-like channel and Na^+ travels paracellularly into the luminal space (**Fig. 1**; Silva et al., 1977).

Rectal gland function works in concert with renal and branchial tissue to regulate salt balance in the marine environment. The activity of the gland is intermittent in nature, likely increasing during periods of salt/volume loading with homeostatic balance of NaCl in elasmobranchs during periods of quiescence most probably regulated at other osmoregulatory organs, as excision or ligation of the rectal gland does not appear to influence NaCl balance in starved elasmobranchs *in vivo* (Wood and Wright, 2016).

Changes in the vascular architecture and secretion rate have been demonstrated in the rectal gland of *S. canicula*, where increased perfusion and secretion rates occurred in animals acclimated to hyposmotic environments, while the opposite occurred in the rectal glands of animals adapted to hyperosmotic environments. In essence, the rectal gland is "switched on" in response to volume loading to dump excess salt and, reduce plasma osmolality, thereby reducing the passive entry of water into the animal. One might predict a similar response following ingestion of a salty meal; however, direct measurement of rectal gland secretion post-feeding in elasmobranchs has yet to be reported, but metabolic enzyme activity to fuel function and mRNA abundance of key transporters both increase in the rectal gland of post-fed *S. suckleyi* (Wood and Wright, 2016).

In FW potamotrygonidae rays the rectal gland is a vestigial organ; however, in the amphihaline bull shark the rectal gland is very much present in FW. This is somewhat of an enigma as one might reasonably predict the FW adapted bull shark would want to retain Na^+ and Cl^- and not excrete it via an active rectal gland. Consequently, the rectal gland of FW adapted bull sharks is somewhat vestigial in nature, with reduced vascularity (and as a consequence, blood flow) and reduced Na^+/K^+-ATPase activity in comparison to the rectal gland in SW adapted fish (Pillans et al., 2008).

The rectal gland has been the subject of a significant amount of research in regard to hormonal control. Given the intermittent nature of the gland's activity and the link to feeding, it is perhaps not surprising that a number of gut derived hormones, alongside those involved in volume regulation, have been examined. Indeed, the most complete description of hormonal control involves the vasodilatory hormone CNP and the intestinal hormone vasoactive intestinal peptide (VIP or Scyliorhinin II in some species). The current working hypothesis is that when an elasmobranch ingests food and/or seawater there is a transient increase in blood volume. This increase in blood volume leads to a release of CNP from the heart, which in turn stimulates rectal gland activity directly through vascular and epithelial routes and indirectly through the mobilization of the intestinal hormone VIP.

Gill

Chondrichthyan fishes usually have five pairs of gills, although there are a few elasmobranch species with six or seven. The gills of chondrichthyan fishes are characterized by the presence of an interbranchial gill septa that extends from the base of the gill arch to the skin, forming distinct external gill slits (Wilson and Laurent, 2002). Each gill arch is made up of lateral rods of cartilage supporting the gill septum, and a sheet of muscular and connective tissue. The dorsal and the ventral surfaces of each gill filament have a row of secondary lamellae, which are the principal sites of gas exchange. The proposed functions of the gill septum are to provide physical support for the gill filaments, which aids in maintaining appropriate direction of water flow across, and blood flow through, the filaments for gas exchange (Olson and Kent, 1980). The cavernous inter-filament space is called the "water canal", and a countercurrent system for effective gas exchange has been proposed between blood and water.

In teleosts, the body of evidence indicates that the gills are the most important site of ionoregulation, as well as gas exchange with the external environment. Gill ionocytes are responsible for ion absorption in the hyposmotic FW environment and ion secretion in the hyperosmotic SW environment (Evans et al., 2005). The ionocytes are characterized by the presence of numerous mitochondria and an extensive tubular system developed in the cytoplasm of these epithelial cells. Their function has been determined by the localization of ion-transporting proteins in the plasma membrane (**Fig. 1**). Although ionocytes have also been found in elasmobranch gill epithelia, their function may be different from those of teleosts (Wilson and Laurent, 2002; Evans et al., 2005). Morphological investigations of marine elasmobranch ionocytes have shown several common features to those of FW teleosts. In the marine elasmobranch gills, the basolateral membrane of the ionocyte forms moderate infoldings but does not appear to have a prominent tubular system (Wilson et al., 2002). Further, a multicellular complex of ionocytes with an accessory cell as has been described in teleosts, but has not been found in elasmobranchs. The apical membrane of SW elasmobranch ionocytes is characterized by the presence of microvilli, which are the characteristics of the salt-absorbing ionocytes in the FW teleost gills (Evans et al., 2005).

Molecular and histochemical investigations of ion-transporting proteins have revealed that there are two main types of ionocytes in the elasmobranch gills: type-A Na^+/K^+-ATPase-rich ionocytes, and type-B vacuolar-type H^+-ATPase (V-ATPase)-rich ionocytes. The type-A ionocytes express Na^+/H^+ exchanger isoform 3 (NHE3) on the apical membrane, which is associated with acid secretion (Tresguerres et al., 2005). Conversely, HCO_3^- excretion has been proposed for the type-B ionocytes, since a Pendrin-like Cl^-/HCO_3^- exchanger is located in the apical membrane of these cells (Piermarini et al., 2002). Therefore, a major role for the ionocytes of marine elasmobranchs is likely acid-base regulation (**Fig. 1**) (Wood and Wright, 2016). This hypothesis is supported by the findings that, in Pacific spiny dogfish, *S. suckleyi*, the protein levels of Na^+/K^+-ATPase and V-ATPase were elevated in the gills following infusion of HCl and $NaHCO_3$, respectively.

It has been frequently cited that in regard to Na^+ and Cl^- balance, the rectal gland is the primary site of NaCl excretion, and the gills of marine elasmobranchs play a minor role. In support of this claim, acclimation of euryhaline stingray and bull shark from FW to SW had no effect on branchial Na^+/K^+-ATPase activity (Wood and Wright, 2016), but Na^+/K^+-ATPase activity was significantly increased in the rectal gland of the bull shark. Furthermore, there was no evidence to support Na^+ or Cl^- excretion from the gills following intravenous infusion of NaCl in the marine adapted *S. suckleyi*, despite no change in plasma osmolality and Na^+ or Cl^-

concentrations in the same individuals (Tresguerres et al., 2005). Lastly, mRNA abundance of *cftr1* was absent and *nkcc1* reduced in comparison to the rectal gland of SW adapted Japanese banded houndshark, *Triakis scyllia* (Takabe et al., 2016).

However, these studies must be balanced with the knowledge that when both the urinary system and rectal gland of the striped cat shark, *Poroderma africanum*, were ligated, the shark was still able to regulate internal levels of NaCl to normal concentrations (Haywood, 1975). The removal of the rectal gland also did not affect the number of type-A ionocytes and Na^+/K^+-ATPase activity in gills of the spiny dogfish, *Squalus acanthias* (Wilson et al., 2002). Further, in starved quiescent *S. acanthias*, rectal gland activity is non-existent but the animal is still able to maintain regulation of NaCl (Wood and Wright, 2016). This suggests the gills are likely acutely involved in balancing Na^+ and Cl^- in the absence of a functional rectal gland. In addition to the type-A and type-B ionocytes, the cell aggregates, named follicularly-arranged Na^+/K^+-ATPase-rich cells (follicular NRCs), were found in the Japanese houndshark gills. The follicular NRCs were characterized by well-developed microvilli on the apical membrane and express Na^+/H^+ exchanger 3 and Ca^{2+} transporter 1, suggesting that the follicular NRCs may serve as absorptive ionoregulatory cells. Thus, it is probable that the gills play a significant role in NaCl balance in marine elasmobranchs; however, the function of branchial Na^+ and Cl^- excretory mechanisms remains unresolved.

Conversely, a number of studies examining the role of the gills in FW adapted elasmobranchs concluded they do contribute to ion uptake, much in the same way as described for FW teleosts (Evans and Claiborne, 2009). Activity of Na^+/K^+-ATPase and V-ATPase was significantly higher in the gills of FW acclimated *H. sabinus* in comparison to SW acclimated individuals (Piermarini and Evans, 2001). Similarly, acclimation of *T. scyllia* from SW to 30% SW resulted in an increased mRNA abundance of *nka* and *nhe3* in the type-A ionocytes (Takabe et al., 2016). Furthermore, gene expression of the sodium chloride co-transporter (*ncc*) was found in a subpopulation of the type-B ionocytes, and mRNA abundance of both *v-atpase* and *ncc* was significantly greater in the 30% SW acclimated fish compared to the 100% SW fish. In the type-A ionocytes, low intracellular sodium concentrations caused by basolateral Na^+/K^+-ATPase would be favorable for apical Na^+ uptake from the environment in exchange for H^+ excretion (Evans et al., 2005). Similarly, basolateral V-ATPase actively pumps H^+ across the basolateral membrane, creating a high intracellular HCO_3^- concentration that is favorable for apical HCO_3^- secretion and chloride ion uptake via a Pendrin-like exchanger. These results indicate that elasmobranch gill ionocytes contribute to NaCl uptake, in addition to the already described function of acid base regulation.

Branchial epithelia in all fish occupy a huge surface area to optimize gas exchange. This combined with the large concentration gradient of urea between the internal and surrounding environments results in the gills being the major site of urea loss in marine elasmobranchs. Despite this huge outwardly directed gradient, the elasmobranch gill has a much lower permeability to urea than marine teleosts. The basolateral membrane of the gill epithelia in a marine adapted *S. suckleyi* has some of the highest cholesterol to phospholipid molar ratios compared to other fishes, which likely lends to the decreased permeability for urea (Hill et al., 2004). Further, these biochemical features are combined with molecular transport systems such as the putative Na^+-coupled urea back transport system in the basolateral membrane (Fines et al., 2001) which may also work to limit urea loss. However, how these mechanisms function is currently unknown, as despite the demonstration that urea transport (UTs) proteins are highly expressed in the kidney, one has yet to be described in the elasmobranch gills, although a UT3 has been shown to be expressed in the gills of the spotted ratfish, *Hydrolagus colliei* (Anderson et al., 2012).

Currently, no studies have examined hormonal control of gill epithelial cell function in elasmobranchs; however, it is reasonable to assume that hemodynamics of gill perfusion would be influenced by vasoactive hormones such as Ang II, AVT and CNP.

Intestine

The nitrogen necessary to synthesize urea and support the ureosmotic strategy of elasmobranchs comes primarily from the digestion of food and subsequent uptake along the gastrointestinal (GI) tract, as well as environmental ammonia uptake across the branchial tissues (Wood and Giacomin, 2016). Despite their carnivorous diet which provides access to exogenous nitrogen, marine elasmobranchs are considered nitrogen-limited (Wood, 2001). This is due in part to their putative intermittent feeding habits (Jones and Geen, 1977) and their reliance on nitrogen for both osmoregulatory processes and somatic growth.

The acquisition of dietary nitrogen occurs primarily across the spiral valve in marine elasmobranchs (Liew et al., 2013). The spiral valve, or intestine, is a compact tube-like organ consisting of between 2 and 50 internal radiating folds. The morphological structure of the internal folds can be either columnar, funnel (orientated toward the anterior or posterior), or scroll-like. Interestingly, the structure of the spiral valve is not correlated with diet type, or limited to phylogenetic order, and different families in one order may have different spiral valve morphologies (Leigh et al., 2021). To compensate for the short length, the internal folds effectively increase the intestinal surface area while also increasing the transit time of the digestive chyme (Leigh et al., 2021), allowing for increased nutrient acquisition.

To better understand the role of the spiral valve in nitrogen acquisition in marine elasmobranchs, research has begun to investigate the movement of urea and ammonia across the GI tract, and the mechanisms involved. In fed spiny dogfish, the uptake of urea along the length of the GI tract was demonstrated using *in vitro* gut sac preparations (Liew et al., 2013). From this work, the spiral valve was shown to take-up significantly more urea than the cardiac and pyloric stomachs, and the colon (Liew et al., 2013). In contrast, the gut sacs from fasted dogfish showed a net efflux (i.e., accumulation) of urea within the lumen of the spiral valve which did not occur in the gastric or colon sacs (Liew et al., 2013). This fed-influx and fasted-efflux of urea matched *in vitro* Ussing chamber flux studies measuring the net movement of ^{14}C-urea across individual spiral valve folds (Anderson et al., 2010). Analysis of plasma

from fed dogfish demonstrated a significant influx of urea into the plasma at 20 h post-feeding (Wood et al., 2007). At the same time, urea concentrations within the spiral valve lumen were less than 100 mmol lower than the plasma (Wood et al., 2007). As the ingested meal contained minimal urea (~4 mmol) compared to the concentrations in the dogfish plasma (~400 mmol), it was suggested that the onset of digestion stimulated an efflux of urea into the spiral valve lumen to balance the osmotic pressure of the plasma to that of the digestive fluids and chyme (Wood et al., 2007). Due to the importance of urea retention for osmoregulation, the subsequent urea influx measured at 20 h post-feeding was likely a strategy to retain urea from fecal excretion. Indeed, when urea concentrations were measured in the colonic fluids of three marine elasmobranchs (*Chiloscyllium plagiosum, L. erinacea, Raja eglanteria*), they were shown to be significantly lower than plasma and spiral valve concentrations (Anderson et al., 2010), indicating high urea uptake and conservation across the preceding spiral valve tissues.

Due to the ureosmotic nature of marine elasmobranchs, research investigating the osmoregulatory strategy of these animals has historically focused on the acquisition, synthesis, and retention of urea. Comparatively, similar research into the acquisition and trafficking of ammonia across the GI tract is currently lacking, despite its role as a precursor for the synthesis of urea via the OUC (Anderson, 1991). To better understand the extent and importance of ammonia uptake across the spiral valve, Hoogenboom and Anderson (2023) fed dogfish a fish-slurry containing ^{15}N-ammonia to track the uptake and use of dietary nitrogen. They demonstrated that within 20 h of feeding, the dietary ^{15}N-ammonia had moved along the GI tract and reached the spiral valve, been acquired by the epithelial tissues, and moved into the plasma for circulation throughout the whole-body. This study showed dietary ammonia was used to synthesize other nitrogen compounds, including urea, glutamine, amino acids, and proteins in various tissues including the spiral valve, liver, and skeletal muscle. Despite the toxic nature of ammonia, the acquisition and use of dietary ammonia is clearly an important component of the nitrogen budget for these animals.

In vitro flux studies have investigated the movement of ammonia across the GI tract. In fed dogfish, a net accumulation (i.e., efflux) of ammonia occurred within the lumen of the cardiac and pyloric stomachs, spiral valve, and colon, as well as the spiral valve of fasted dogfish, but no influx of ammonia was demonstrated (Wood et al., 2019). In a follow-up study that examined if the digestion of supraphysiological concentrations of dietary urea (700 mM) affected the trafficking of urea and ammonia across the dogfish GI tract, *in vitro* gut sacs showed an accumulation of ammonia in the intestinal lumen (~4 mM), but also a significant influx of ammonia (~0.7 mM) to the serosal medium (representative of the blood plasma) (Hoogenboom et al., 2020). In a third study, individual spiral valve folds mounted in Ussing chambers demonstrated a significant net influx of ^{14}C-methylamine (an analog for ammonia) into the lumenal medium, but no apparent accumulation/efflux in the serosal medium (Hoogenboom and Anderson, 2023). Despite the conflicting results between these three studies, collectively they indicate the capacity of bidirectional trafficking of ammonia across the spiral valve.

Molecular studies investigating the transcript abundance and localization of urea and ammonia transport proteins have also begun to shed light on the nature of nitrogen movement and acquisition (Anderson et al., 2010; Hoogenboom and Anderson, 2023; Hoogenboom et al., 2023; Nawata et al., 2015). The mRNA of a urea transporter (UT) was identified in the spiral valve of the little skate (Anderson et al., 2010) and the spiny dogfish (Nawata et al., 2015), and showed no change in transcript abundance in the spiny dogfish between fed and fasted animals (Hoogenboom and Anderson, 2023). In the cloudy catshark (*Scyliorhinus torazame*), the UT transcript abundance differed between fed and fasted animals, with the anterior spiral valve region of fed catshark having lower levels than the anterior region of fasted catshark (Hoogenboom et al., 2023). Immunohistochemistry identified the localization of the UT along the basolateral membrane of the spiral valve epithelial cells and intestinal blood vessels (Hoogenboom et al., 2023). The function of this basolateral UT is presumably to shuttle urea between the plasma and intestinal epithelial cells, to facilitate the efflux of urea at the onset of digestion and its subsequent reacquisition at 20 h post-feeding, as described by Wood et al. (2007), as well as any urea acquired through the digestion of a meal. It is also likely responsible for moving urea synthesized within the intestinal epithelial cells via the OUC (Kajimura et al., 2008; Hoogenboom and Anderson, 2023; Hoogenboom et al., 2023).

Two ammonia transporters (Rhbg and Rhp2) were also identified within the spiral valve of the spiny dogfish (Nawata et al., 2015; Hoogenboom and Anderson, 2023), the cloudy catshark (Hoogenboom et al., 2023), and the little skate (Anderson et al., 2010). Rhbg showed no difference in transcript abundance between fed and fasted states in both the dogfish and the catshark. Rhp2 showed significantly lower levels in fed catsharks compared to fasted, while the dogfish showed significantly higher levels in the mid spiral valve region of fed animals compared to fasted (Hoogenboom and Anderson, 2023; Hoogenboom et al., 2023). Immunohistochemistry showed the localization of Rhp2 along the apical brush border membrane of the intestinal epithelial cells, which would facilitate the movement of ammonia between the lumen of the spiral valve and the epithelial cells (**Fig. 1**) (Hoogenboom et al., 2023). The spiral valve is likely the primary site of nitrogen uptake in the intestine and plays a critical role in the overall osmoregulatory strategy, given the reliance on urea as an osmolyte. Linked to this strategy is the microbiome of the fish which most likely aids in the breakdown of macronutrients and the availability of ammonia for uptake and subsequent incorporation into urea. The exact mechanisms for this interaction remain to be determined but are an exciting avenue for future research.

In addition to the important role of urea balance, the intestine is also critical for salt and water balance. Drinking the environment is a well-established necessary physiological response for marine teleosts. The iso- or slightly hyperosmoregualtory strategy of marine elasmobranchs means there is less, or even no, requirement to drink the environment as part of their osmoregulatory strategy. However, numerous species of elasmobranch are known to move between salinities (Martin, 2005) and therefore would likely need to drink the environment to counter volume loss as they move from a hypo-to a hyperosmotic environment. Indeed, transfer of 80% SW acclimated *S. canicula* to 100% SW resulted in a transient rise in plasma NaCl concentration but not urea, suggesting that the increase in solute concentration was not simply the result of osmotic water loss. Subsequent studies demonstrated that the source of the additional Na^+ and Cl^- ions was likely imbibed SW, and a drinking response has been described in at least two other elasmobranch species (Anderson et al., 2001; De Boeck et al., 2001).

Numerous gastroenteropancreatic hormones have been identified in the elasmobranch intestine using pharmacological and molecular tools; however, few studies have examined the functional role of these hormonal systems regarding osmoregulation. Indeed, there are no studies that have examined hormonal control of urea uptake across the intestine, although hormonal regulation of drinking has been described (Anderson et al., 2001). The renin angiotensin system is recognized as one of the most potent regulators of drinking in mammals. Interestingly, it appears that this is a highly conserved feature of this hormone system, as Ang II was also shown to stimulate a drinking response in *S. canicula*, and this drinking response was inhibited by the antidipsogenic hormone CNP.

Conclusion

In this article we have discussed the unusual osmoregulatory strategy of the elasmobranchs and holocephalans. Urea is expensive to make (5 ATP) and a metabolic dead-end, so the challenge of maintaining a high concentration of urea for osmoregulation has necessitated the evolution of a number of morphologically and physiologically complex systems to support this nitrogen-limiting and energetically-expensive tactic. The liver is a major site of urea synthesis, but extrahepatic synthesis likely supports development of appropriate gradients across epithelia to limit urea loss in the kidney and intestine. Nephron structure and overall renal architecture is probably the most complex in the vertebrate phyla, all geared toward urea retention. Modifications to gill function and an almost complete absence of urea in rectal gland fluid suggest a significant scavenging mechanism to retain urea at these ion-regulating organs, and intestinal retention is strongly linked to dietary uptake and likely connected to biome function. The rectal gland is probably the most well-studied elasmobranch organ and tightly linked to excretion of transient increases in Na^+ and Cl^- following feeding, with the gills playing the major role during times of rest; however, NaCl excretory routes via the gills have yet to be described. There are numerous avenues of exploration to further our understanding of this unusual osmoregulatory strategy in this enigmatic group of fishes.

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See Also: Introduction to elasmobranch physiology; Osmoregulation in fishes: An introduction; Salt secretion by the gill of marine teleost fishes; The kidney for osmoregulation.

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