博士論文（要約）

Studies on the kidney function in environmental adaptation of euryhaline bull shark, *Carcharhinus leucas*

広塩性軟骨魚オオメジロザメの環境適応における腎機能の研究

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General Introduction

Cartilaginous fishes and their strategy of body fluid regulation

The class Chondrichthyes is commonly known as cartilaginous fishes, and it contains elasmobranchs (sharks and rays) and holocephalans (chimaeras and elephant fishes) (Compagno, 1999). There are 1,185 species of the living cartilaginous fishes recognized (White and Last, 2012). Cartilaginous fishes are among the apex predators in the marine ecosystem (Lack and Sant, 2009), and their species conservation and population management are important for the sustainability. According to the IUCN's Red List of Threatened Species, approximately 17% of elasmobranch species are classified as Critically Endangered, Endangered or Vulnerable species, and 13% are considered as Near Threatened, while 47% of cartilaginous fish species are listed as Data Deficient (Polidoro et al., 2008). One cause for such critical situation is overfishing including bycatch and shark finning. In addition to such human threat, many unique features of cartilaginous fishes make conservation and restoration difficult. For instance, their life cycles are generally longer than those of teleosts as their development, growth, maturation and gestation take years to complete. All cartilaginous fishes perform internal fertilization and lay few fertilized eggs or give birth to few juveniles when compared to the cases in teleosts. Therefore, restoration of the population is rather difficult when the decrease in population happens. It is also difficult for researcher to study the biology of cartilaginous fishes as many are pelagic species that are not suitable in captive environments. Therefore, many of their physiological features are still at the beginning of investigation.

All extant holocephalans and most (approximately 95%) elasmobranchs are principally marine species (Ballantyne and Fraser, 2013; Lucifora et al., 2015), and their osmoregulatory strategy is also different from those of teleosts. Marine teleosts maintain their plasma osmolality around 300 mOsm, thus they have the risk of dehydration. To overcome this problem, marine teleosts drink seawater and absorb water from the intestine, while they

excrete excess NaCl and divalent ions from the gill and the kidney, respectively (Evans and Claiborne, 2009). Meanwhile, marine cartilaginous fishes evolve a different strategy to acclimate to the high-osmolality in the marine environment by keeping their plasma osmolality slightly higher than that of environmental seawater. This high plasma osmolality is achieved by the accumulation of nitrogenous compounds such as urea and methylamine in the body (Hazon et al., 2003). Urea is the predominant osmolyte in cartilaginous fishes, and plasma concentration of urea range from 350 to 450 mM, which accounts for approximately one-third to a half of the plasma osmolality. As a result, cartilaginous fishes do not suffer from dehydration in SW (Smith, 1936; Robertson, 1975; Hazon et al., 2003; Hyodo et al., 2004b).

Osmoregulatory organs of cartilaginous fishes: kidney is one of the important organs for body-fluid homeostasis

To achieve the urea-based osmoregulation, various osmoregulatory organs, such as gill, rectal gland, and kidney, work in harmony in cartilaginous fishes. In marine teleosts, gill is important for excrete excess amount of NaCl via ionocytes (Evans and Claiborne, 2009). However, NaCl excretion is not prominent in cartilaginous fish gill, because the known NaCl excretory machinery including Na^+-K^+-$2Cl^-$ cotransporter type-1 (NKCC1, Slc12a2) and cystic fibrosis transmembrane conductance regulator (CFTR) are not found in their branchial ionocytes. Instead, ionocytes in cartilaginous fishes have been suggested to play a role in acid-base balance. Co-localization of apical Cl^-/HCO_3^- exchanger (pendrin) and basolateral vacuolar type H^+-ATPase (VHA) is likely responsible for base secretion, while apical Na^+/H^+ exchanger type 3 (NHE3) and basolateral Na^+/K^+-ATPase (NKA) is likely crucial for acid secretion in Atlantic stingray (Piermarini et al., 2002; Choe et al., 2007). Meanwhile, rectal gland is a well-recognized NaCl-secretory organ in cartilaginous fishes (firstly reported in

Burger and Hess, 1960). The rectal gland is composed of mitochondria-rich cells that excrete isosmotic NaCl solution to the lumen, which eventually direct NaCl solution into the rectum for excretion. The mitochondria-rich cells of rectal gland express NKA, NKCC1, CFTR, and K^+ channel (KCNQ1), which resemble the excretory machinery of NaCl reported in the branchial ionocytes of marine teleosts (Xu et al., 1994; Silva et al., 1996; Waldegger et al., 1999; Evans and Claiborne, 2009; Wright and Wood, 2013).

In cartilaginous fishes, kidney is another important organ for body-fluid homeostasis. Plasma urea is freely filtered by the glomerulus and, in mammals, the filtered urea is concentrated in the urine for excretion. However, urea is valuable osmolyte in cartilaginous fishes. Therefore, cartilaginous fish kidney reabsorbs more than 90% of the filtered urea from primary urine to reduce the urea loss (Smith, 1936; Kempton, 1953; Boylan, 1967). Because of the urea-reabsorption function, the nephrons of marine cartilaginous fishes are highly elaborate and possess unique features that are not found in other vertebrates (Lacy and Reale, 1985; Hentschel et al., 1998; Hyodo et al., 2014). The kidney of cartilaginous fishes is a dorsoventrally flattened organ located in the caudal part of the abdominal cavity, and composed of multiple, irregularly shaped lobules. Each lobule is separated into two distinct regions: a sinus zone and a bundle zone. Renal corpuscles (RC) are situated at the boundary between the two zones. The sinus zone occupies a larger region in the kidney, wherein renal tubules are individually exposed to the blood sinuses. On the other hand, renal tubules in the bundle zone are densely packed. Each nephron has tubule that makes four turns, and passes sequentially from bundle to sinus zone twice. Starting from a RC, the first and third (early distal tubule, EDT) loops are situated in the bundle zone, while the second (proximal II) and fourth (late distal tubule, LDT) loops are located in the sinus zone. The final segment (collecting tubule, CT) is also located in the bundle zone, and is connected to the collecting duct (Fig. I; Hyodo et al., 2014). In the

bundle zone, the resulting five tubules originated from a single nephron (the descending and ascending limbs of the first and the third loops and the collecting tubule) are enclosed in a sac-like peritubular sheath. The cells of the sheath are connected by tight junction, suggesting that the sheath acts as a barrier to form a separate microenvironment in each bundle (Lacy and Reale, 1986).

Functions of each nephron segment were investigated by mapping of various pumps, channels and transporters in the nephron (Hyodo et al., 2004a; Yamaguchi et al., 2009; Kakumura et al., 2015; Hasegawa et al., 2016). Based on the results of molecular mapping, the mechanism of urea reabsorption in the kidney of cartilaginous fishes was modeled (Fig. II; Hyodo et al., 2014). In this model, *1*) prominent active transports of NaCl from the lumen to peritubular sheath are driven by NKA and Na^+-K^+-$2Cl^-$ cotransporter type-2 (NKCC2, Slc12a1) in the early distal tubule. *2*) This results in an increased osmolality inside peritubular sheath that initiates passive absorption of water from the first loop in the bundle. As a result, a low-urea environment is generated within the peritubular space. *3*) Because of the elastic nature of the peritubular sheath, hydrostatic pressure builds up and drives the low-urea fluid flows from the tip of the bundle to the renal portal system via central vessel. *4*) Urea is reabsorbed from the collecting tubules to the central vessels down a concentration gradient via the facilitative urea transporter (UT, Slc14a2) in collecting tubules.

The importance of bundle structure for urea reabsorption is supported by comparative anatomy of nephron among cartilaginous fishes. Freshwater stingrays do not accumulate urea in the body (Goldstein and Forster, 1971), and their nephrons lack the bundle-like structure (Lacy and Reale, 1999).

Euryhalinity of cartilaginous fishes

Most elasmobranchs (approximately 95%) are principally marine species (Ballantyne and Fraser, 2013; Lucifora et al., 2015), but some species are known to tolerate various degrees of diluted SW, and are sometimes referred as "partially euryhaline" (Hazon et al., 2003). For instance, Japanese banded houndshark (*Triakis scyllium*) can survive salinity transfer from full-strength SW to 30% SW (Yamaguchi et al., 2009). However, only 5% of elasmobranchs can enter freshwater (FW) environment, which is in contrast to the case in teleost fishes (more than 40% of known species can enter FW) (Ballantyne and Robinson, 2010; Lucifora et al., 2015). Ballantyne and Fraser (2013) reported that less than 0.5% of elasmobranchs are truly euryhaline species, which can survive in both SW and FW environments.

On the other hand, stenohaline FW stingrays exist (Lovejoy et al., 1998; Ballantyne and Robinson, 2010), although there are only a few species. Interestingly, FW stingrays of the Family Potamotrygonidae do not accumulate urea in the body (Thorson et al., 1967; Thorson, 1970; Gerst and Thorson, 1977; Thorson et al., 1978), and their plasma osmolality ranged from 300-400 mOsm (Ogawa and Hirano, 1982; Wood et al., 2002), which is comparable with those of FW teleosts. Since FW stingrays do not have ability to accumulate urea, they cannot tolerate to high salinity environment.

In the case of euryhaline teleost fishes such as salmonids and eels, it is well-recognized that they have osmoregulatory mechanisms in SW, but they also develop hyper-osmoregulatory mechanisms when they migrate to FW environments. A typical example of osmoregulatory mechanisms is the shift in branchial ionocytes. In SW environment, branchial ionocytes express basolateral NKCC1 and apical CFTR to excrete excess NaCl via active transport system driven by NKA. When euryhaline teleosts enter FW, two-types of ionocytes express either apical Na^+/H^+ exchanger 3 (NHE3) or Na^+-Cl^- cotransporter (NCC) develop to absorb NaCl from the

ion-poor environment. In SW, a teleost kidney excretes divalent ions in a small volume of urine, whereas a kidney produces a large volume of diluted urine to excrete excess water in FW environment.

How about euryhaline elasmobranchs? Assuming that euryhaline elasmobranchs also switch their body-fluid regulation system between SW- and FW-types, can euryhaline elasmobranchs conduct urea-based osmoregulation in SW, while eliminate urea production and retention in FW as in stingrays? The fact is that euryhaline elasmobranchs possess high plasma NaCl and urea (osmolality approximately 600 mOsm) even in FW, which is nearly twice of the plasma osmolality of FW teleosts. Why do they keep such high levels of NaCl and urea? and how? The body-fluid homeostasis of euryhaline elasmobranchs is an important issue to be studied.

Previous studies showed that the size of rectal glands of bull sharks and Atlantic stingrays was smaller in FW than in SW (Oguri, 1964; Piermarini and Evans, 1998). NKA activities of rectal glands were lowered in FW for both bull sharks (Pillans et al., 2008) and Atlantic stingrays (Piermarini and Evans, 2000). The same phenomenon was also observed in leopard sharks *Triakis semifasciata* transferred to diluted SW (Dowd et al., 2010). In the gill, NKA activity was higher in FW- than in SW-reared Atlantic stingray (Piermarini and Evans, 2000). Upregulation of NKA and NHE3 was also reported in bull sharks reared in FW, implying the gill ionocytes may contribute to Na^+ uptake (Reilly et al., 2011). In Atlantic stingrays, glomerular filtration rate and urine flow rate were increased following acute transfer to 50% SW (Janech et al., 2006), and the urine flow rate was much higher than that of FW teleosts (Smith, 1931; Janech and Piermarini, 2002). The increase in NKA activity was also reported in the kidney of bull shark transferred to FW (Pillans et al., 2005), implying that ion transports are more active in FW to produce copious diluted urine. However, most studies were fragmentary,

and the mechanisms of FW acclimation in cartilaginous fishes remain to be clarified.

Bull shark *Carcharhinus leucas*-an euryhaline elasmobranch

Bull shark (*Carcharhinus leucas*; the order, *Carcharhiniformes*, the family, *Carcharhinidae*) is one of the euryhaline cartilaginous fishes including sawfishes (*Pristis microdon* and *P. perotteti*) and Atlantic stingray (*Hypanus sabina*) (Ballantyne and Fraser, 2013). They inhabit tropical and subtropical waters of continental shelf at a depth of about 150 m, estuarine and freshwater in a world distribution (Simpfendorfer and Burgess, 2009). Carlson and coworkers (2010) revealed that bull sharks in the Gulf of Mexico travelled an average of 143.6 km per 85 days with the greatest 1506 km by using pop-up satellite archival tags. Temperatures of waters in which it regularly occurs are 18°C to 28°C and it normally prefers water above 20°C (Compagno, 1984). The diet is relatively diverse, including turtles, birds, dolphins, terrestrial mammals, crustaceans, echinoderms, teleosts and elasmobranchs (Last and Stevens 2009). However, the most commonly eaten prey items are teleosts and elasmobranchs. Reproduction is by placental viviparity. Martínez and coworkers (2004) estimated that age-at-maturity of bull sharks in Southern Gulf of Mexico is 10 years [total length (TL) = 204 cm] for females and 9-10 years (TL = 190-200 cm) for males. They also estimated that the oldest female is 28 (TL = 256.0 cm), and the oldest male was 23 (TL = 243.0 cm). The gestation period is around 10-11 months, with usually 1-13 pups birth in late spring and summer (Castro, 1993; Compagno, 1984). Parturition may occur year-round in warm areas such as Nicaragua (Castro, 1993), but the length of their reproductive cycle indicates that breeding may occur biennially (Compagno, 1984). Bull shark inhabits coastal, offshore areas, estuary and river, where their natural habitats and breeding grounds are susceptible to be affected human activities. Therefore, bull shark is categorized as a Near Threatened species according to the IUCN's Red List of

Threatened Species (Simpfendorfer and Burgess, 2009).

Pioneer studies of bull sharks in Lake Nicaragua and subsequent works in other areas have established some of their physiological, ecological and behavioral aspects in a FW environment (Thorson, 1962; Thorson et al. 1966; Thorson, 1971). Bull sharks spend most of their lifetime in SW, but they live in a wide range of salinities throughout their lifecycle (Thorson, 1971; Montoya and Thorson, 1982; Simpfendorfer et al., 2005; Carlson et al., 2010). Female bull sharks are thought to give birth in estuaries or river mouths, and neonates and juveniles are thought to migrate upstream to occupy inshore rivers as nursery habitats (Ortega et al., 2009; Werry et al., 2012). Exploration of FW habitats by juvenile bull sharks has been reported in the southern United States (about 30km from estuary in Simpfendorfer et al., 2005; about 20km in Ortega et al., 2009; about 30km in Matich and Heithaus, 2015), central America (about 200km in Thorson, 1971; about 70km in Montoya and Thorson, 1982), Australia (about 70km in Pillans and Franklin, 2004; about 20km in Werry et al., 2012) and South Africa (1120km in Cliff and Dudley, 1991; about 20km in McCord and Lamberth, 2009). Recently, a worldwide records of bull sharks in FW was compiled by Gausmann (2018). Once bull sharks reach sexual maturity, they move from riverine and estuarine to coastal and offshore areas for further growth and breeding (Curtis et al., 2011).

Bull sharks and other euryhaline elasmobranch species maintain high internal NaCl and urea in FW (Janech and Piermarini, 2002; Pillans and Franklin, 2004). Therefore, it is likely that bull sharks face severe water gain and osmolyte loss in FW via branchial diffusion and urine. To counter these problems, that the kidney of bull shark must excrete excess water due to the osmotic influx in FW, while concomitantly retaining ions and urea by enhanced reabsorption. Therefore, the author focused on the kidney because this is the only organ capable of excreting excess body water and it plays an important role for regulating body fluid contents

and osmolality. The author hypothesized that the expression of key proteins involved in osmoregulation is altered in the kidney when bull sharks move from SW to FW habitats. So, the author focused on the genes in solute carrier (Slc) families and the epithelial Na^+ channels in the kidney because these genes are involved in reabsorbing or excreting osmolytes in the vertebrate kidneys.

Solute carrier family genes

Membrane proteins that contribute to transportation of substrates have roughly been divided into several categories: ATP-powered pumps, ATP-binding cassette (ABC) transporters, ion channels, water channels, and solute carrier family proteins, and so on (Fredriksson et al., 2008; He et al., 2009). ATP-powered pumps and ABC transporters use energy released by ATP hydrolysis to drive substrate movement across membranes against their electric and/or chemical gradients. ATP-powered pumps, such as Na^+/K^+-ATPase (NKA), are called active transporters and create ion/solute gradients across membranes. The resulting electric and/or chemical gradients can be the driving force for subsequent transport of substrates across membrane via secondarily active or passive transporters and channels. In contrast, channels and transporters transport substrates passively (following to electric or chemical gradient). Channels regulate ion or solute flow by the gating mechanisms and the single channel conductance. Transporters typically have a fixed stoichiometry of ion/solute movement per translocation cycle (Hediger et al., 2004). Solute carrier (Slc) family transporters are the largest group of substrates transporting proteins. The Slc family includes 65 gene families in human (SLCtables, http://slc.bioparadigms.org/) or 51 gene families in teleosts (Verri et al., 2012). They control uptake and excretion of crucial substrates such as sugars, amino acids, nucleotides, inorganic ions and drugs passively in various tissues including kidney. In this Ph.D. thesis, the author

focused on Slc12a3 and Slc4a11 in the Slc family genes and epithelial Na^+ channel (ENaC, Sccn1) in the ENaC/Degenerin superfamily based on the results of RNA-sequencing analysis. In the following sections, the current knowledge is briefly described concerning the above proteins.

Slc12 family: Na^+-K^+-$2Cl^-$ cotransporter and Na^+-Cl^- cotransporter

Slc12 family is an electroneutral cation-chloride-coupled cotransporter gene family. Nine genes (Slc12a1-9) belong to this family, and the products SLC12A1-7 have been shown to transport Cl^- together with Na^+ and/or K^+ in an electroneutral fashion (Hebert et al., 2004). The first identified gene in this family was Na^+-K^+-$2Cl^-$ cotransporter 1 (NKCC1; Slc12a2) reported in the rectal gland of spiny dogfish, *Squalus acanthias* (Xu et al., 1994). Secondly, Na^+-Cl^- cotransporter (NCC; Slc12a3) was identified in the urinary bladder of winter flounder, *Pseudopleuronectes americanus* (Gamba et al., 1993).

Two isoforms of NKCC are known: basolaterally-located type-1 (NKCC1, Slc12a2) (Delpire et al., 1994; Xu et al., 1994) and apically-located type-2 (NKCC2, Slc12a1) (Gamba et al., 1994; Payne et al., 1994). Both isoforms transport one Na^+, one K^+, and two Cl^- (Greger et al., 1981; Greger et al., 1983) and are inhibited by loop diuretics such as bumetanide and furosemide (Hebert et al., 2004). In the mammalian kidney, NKCC2 is expressed in the thick ascending limb of the loop of Henle (Mount et al., 1999) to reabsorb NaCl from the glomerular filtrate (Takahashi et al., 2000; Lifton et al., 2001). Because of this feature, the thick ascending limb is also called the “diluting segment”. In FW teleosts, NKCC2 is expressed in the early distal tubules and important for salt retention (Wingert et al., 2007; Kato et al., 2011). In the kidney of SW cartilaginous fishes, NKCC2 is expressed in the early and late distal tubules (Hyodo et al., 2014; Kakumura et al., 2015). On the other hand, NKCC1 is expressed in the

rectal gland of the marine cartilaginous fishes (Xu et al., 1994) and the branchial ionocytes of teleosts in SW environment (Inokuchi et al., 2008; Christensen et al., 2012; Hsu et al., 2014). NKCC1 is a key transporter for excretion of excess salt in marine environment.

Na^+-Cl^- cotransporter (NCC; Slc12a3) is a thiazide-sensitive transporter (Hebert et al., 2004) and is important for renal NaCl retention. Thiazides inhibit NaCl cotransport in the urinary bladder of winter flounder (Stokes, 1984) and in the distal convoluted tubule of the rat kidney (Ellison et al., 1987), resulting in an increase of Cl^- excretion in urine (Kunau et al., 1975). Therefore, the thiazide-sensitive NCC is considered as the major salt reabsorption transporter in the mammalian distal convoluted tubule where 5-10% of the filtered Na^+ and Cl^- is reabsorbed (Hebert et al., 2004). In the kidney of FW teleosts, NCC is expressed in the late distal tubule to reabsorb NaCl to generate hypotonic urine (Kato et al., 2011). NCC is also expressed in the branchial ionocytes to absorb NaCl from environmental water in teleosts acclimated to FW (Hiroi et al., 2008; Inokuchi et al., 2009; Hwang, 2009; Christensen et al., 2012; Hsu et al., 2014). However, it is now recognized that the branchial NCC is encoded by a separate gene, Slc12a10 (also known as NCC2). Since the Slc12a10 clade form a separate lineage from that of Slc12a3 phylogenetically, Slc12a10 is considered as a “teleost-specific” or “ray-finned fish-specific” NCC (Takabe et al., 2016). In cartilaginous fishes, Slc12a3 mRNA is expressed in the branchial ionocytes, and the expression was upregulated in houndsharks acclimated to 30% SW, suggesting that NCC is important for low-salinity acclimation also in cartilaginous fishes (Takabe et al., 2016). However, no NCC study has been conducted in the kidney of cartilaginous fishes.

The epithelial Na^+ channel

The epithelial Na^+ channel (ENaC) is a member of ENaC/ Degenerin superfamily protein. Amiloride-sensitive Na^+ conductance in epithelial cells has been demonstrated by measurements of net fluxes, short-circuit current and conductance (Garty and Palmer, 1997; Alvarez de la Rosa et al., 2000). It was shown that the epithelial sodium channel (ENaC)/ Degenerin gene family represents a new class of ion channels (Kellenberger and Schild, 2002) in addition to the Slc families. The Degenerin genes *deg-1* and *mec-4* were first identified from *Caenorhabditis elegans* (Chalfie and Wolinsky, 1990; Driscoll and Chalfie, 1991). The name degenerin comes from the cellular phenotype induced by mutations in the *deg-1* and its related genes that resulted in selective degeneration of sensory neurons involved in touch sensitivity (Chalfie and Wolinsky, 1990; Kellenberger and Schild, 2002). On the other hand, the cDNA sequences of ENaC subunits were determined in rat and human (Canessa et al., 1994; McDonald et al., 1994; McDonald et al., 1995). ENaC is composed of three subunits (ENaCα or ENaCδ, ENaCβ and ENaCγ) (Firsov et al., 1998). The driving force for Na^+ transport via ENaC is generally produced by basolateral NKA (Kellenberger and Schild, 2002; Kashlan and Kleyman, 2011). ENaC activity is inhibited by amiloride (Garty and Palmer, 1997) and is regulated by the renin‐angiotensin‐aldosterone system (Asher et al., 1996; Bhalla and Hallows, 2008). In the mammalian kidney, ENaC is expressed in the aldosterone-sensitive segments, including distal convoluted tubules, connecting tubules and collecting ducts (Bachmann et al., 1999). When water and salt balance is at normal physiological range, the Na^+ reabsorption rate via ENaC is a small fraction. In response to dehydration and/or salt deprivation, aldosterone and vasopressin stimulates ENaC to increase Na^+ reabsorption. This hormonal feedback control is essential for the maintenance of Na^+ and water balance (Rossier et al., 1994; Schild, 2010).

Teleosts express acid-sensing ion channel (ASIC) in the branchial ionocytes. ASIC

belongs to the ENaC/Degenerin family, but is not the ortholog of ENaC. ASIC is H^+-gated ion channel. In mammals, ASIC is expressed in the central and peripheral nervous systems and have roles in nociception, mechanosensation, fear-related behavior and seizure termination (Chen et al., 1998; Deval and Lingueglia, 2015). ASIC participates in acid-sensing in the gastrointestinal tract and sour taste sensing (Holzer, 2015). It is likely that ENaC was lost in the ray-finned fish lineage, because ENaC genes are absent in all teleost genomes but were identified in lamprey, elephant fish and lungfish (Hwang, 2009; Hanukoglu and Hanukoglu, 2016). In the gill and kidney of lamprey, ENaC may be responsible for Na^+ reabsorption in FW as the expression of ENaC was downregulated after transfer to SW (Ferreira-Martins et al., 2016). Similar results were reported in lungfish (Uchiyama et al., 2012; Uchiyama et al., 2015) and salamander (Uchiyama et al., 2011). Three ENaC subunits (ENaCα, ENaCβ and ENaCγ) have been found from elephant fish genome (Venkatesh et al., 2007), but no functional investigation has been conducted in cartilaginous fishes.

Slc4 family

Slc4 family is a major group of bicarbonate transporters and consists of 10 genes in mammals (Slc4a1-5, 7-11). Eight of Slc4 family members fall into two major groups: (1) Cl^--HCO_3^- exchangers (or anion exchangers; AE1-3) and (2) Na^+-coupled HCO_3^- transporters. The Na^+-coupled HCO_3^- transporters are further divided into electrogenic (NBCe1 and NBCe2) and electroneutral (NBCn1, NBCn2, NDCBE) transporters (Romero et al., 2013). On the other hand, function of Slc4a9 (AE4) and Slc4a11 (BTR1) has not been well-established.

Molecular phylogenetically, Slc4a11 is grouped as a distant member in the Slc4 family (Parker et al., 2001) and was considered as a boron transporter in plants (Takano et al., 2002) or as a Na^+ and borate cotransporter in animals (Park et al., 2004). However, contradictory

results have recently been obtained; Slc4a11 was shown to be a Na^+-coupled OH^- transporter (Jalimarada et al., 2013) or NH_3-H^+ (or NH_4^+) cotransporter (Patel and Parker, 2015). In mammals, Slc4a11 is expressed in the corneal endothelium, basolateral membrane in the thin descending loops of the kidney, and the inner ear (Damkier et al., 2007; Gröger et al., 2010). Loss of Slc4a11 caused the corneal endothelial dystrophy, and polyuria and over excretion of NaCl as the results of urinary osmotic imbalance (Gröger et al., 2010). Meanwhile, Slc4a11 is expressed in the apical membrane of the distal segment in the kidney of the euryhaline pufferfish (*Takifugu obscures*), and its role on borate excretion to urine has been suggested by *Xenopus* oocyte experiment (Kato et al., 2013; Kato, unpublished observation). Borate ion [$B(OH)_4^-$] is the ionized form of boric acid (Dordas et al., 2001; Gröger et al., 2010). Plasma concentration of boric acid (in human, 0.025 μg/L) is considerably lower than that in environments (SW: 4 to 5 mg/L; FW: 20 μg/L) (Green et al., 1994; Vengosh et al., 1999; Nadav, 1999). Although plasma concentration of boric acid is quite low, the lack of boron (boric acid or borate ion in the body) impaired metabolic function, in particular Ca^{2+} and Mg^{2+} homeostasis, and also impaired activity of steroid hormones in the adult zebrafish and human (Nielsen, 2000). On the other hand, high concentration of boric acid has toxic effects on cell growth and proliferation (Park et al.,2004), implying that excess borate ion must be excreted in SW.

The aim of this study

The final goal of this study is to demonstrate euryhaline mechanisms of bull sharks. To achieve this, the author focused on the kidney function, and investigated changes in kidney function in FW-acclimated bull sharks under captive condition. RNA-sequencing and subsequent molecular histochemical analysis (Chapter 1) were performed. The identified genes

from Chapter 1 are used to investigate kidney function of bull sharks naturally inhabiting Urauchi River in Iriomote Island (Chapter 2).

In Chapter 1-1, bull shark samples were used from the previous transfer experiment (SW to FW), and conducted a transcriptomic search for transcripts exhibiting significant increases in expression levels in FW-acclimated bull sharks by RNA-sequencing (RNA-seq). From the differential gene expression profiles, subsequent quantitative PCR, and *in situ* hybridization, it was showed that the expression of Na^+-Cl^- cotransporter (NCC) was substantially upregulated in the late distal tubule (LDT; the 4th loop) and the collecting tubule (the final segment) after the transfer of bull sharks from SW to FW. In contrast, analyzing the houndsharks *Triakis scyllium*, to compare, the distribution and expression levels of NCC were not changed when they were acclimated to 30% SW (N.B. 30% SW is used because houndsharks cannot survive in FW). These findings indicate that the upregulation of NaCl reabsorption by NCC is one of the crucial mechanisms that contributes to the euryhalinity of bull shark.

In Chapter 1-2, the author focused on epithelial Na^+ channel (ENaC) that facilitates salt transport in the kidney. Although the RNA-seq analysis did not show a significant increase in the FPKM (fragments per kilobase of exon per million reads mapped) values of ENaC subunit mRNAs, qPCR analysis showed significant upregulation in the expressions of ENaC subunits. *In situ* hybridization results further revealed that expression of ENaC subunit mRNAs were extended to the collecting tubule (CT) in FW-acclimated individuals. These results suggest that ENaC is an important channel in FW for reabsorption of Na^+ at the LDT and CT segments.

In Chapter 1-3, the author focused on the transcripts exhibiting significant decrease in expression in FW-acclimated individuals. From the RNA-seq analysis, Slc4a11, a putative borate transporter, was identified based on the downregulation. Subsequent qPCR and *in situ* hybridization analyses revealed that Slc4a11 mRNA was highly expressed in the LDT of SW-

individuals, and the expression was downregulated in FW-individuals. Functional analysis of Slc4a11 using recombinant protein expression in *Xenopus* oocytes confirmed that Slc4a11 transports borate specifically. Following the acclimation to FW, transporter expression for NaCl reabsorption was upregulated, while transporters for ion excretion (e.g., Slc4a11) was downregulated, suggesting that the LDT is a key segment for fine-tuning the functions of the nephrons.

In Chapter 2, the field survey of bull sharks was conducted in Urauchi River of Iriomote Island. Capture survey was performed using gill nets and concomitant investigation of environmental parameters of the river. The survey revealed that all captured bull sharks in this river were juveniles. When juveniles were caught, salt wedge was always observed in the river; juveniles were not caught when the river water was completely FW vertically. This finding implies that bull sharks prefer brackish water in the river. The NCC expression in the kidney of juveniles caught in the river was in between those of SW- and FW-acclimated bull sharks shown in Chapter 1-1. The idea of salt-wedge preference is reasonable because river bull sharks maintain high plasma NaCl and urea levels. Even in the river, seawater invades from the bottom layer, resulting in a brackish environment that euryhaline bull shark exploit.

In the General Discussion, the results of all chapters are discussed and proposed a model showing the special renal mechanisms that enable euryhalinity in bull shark.

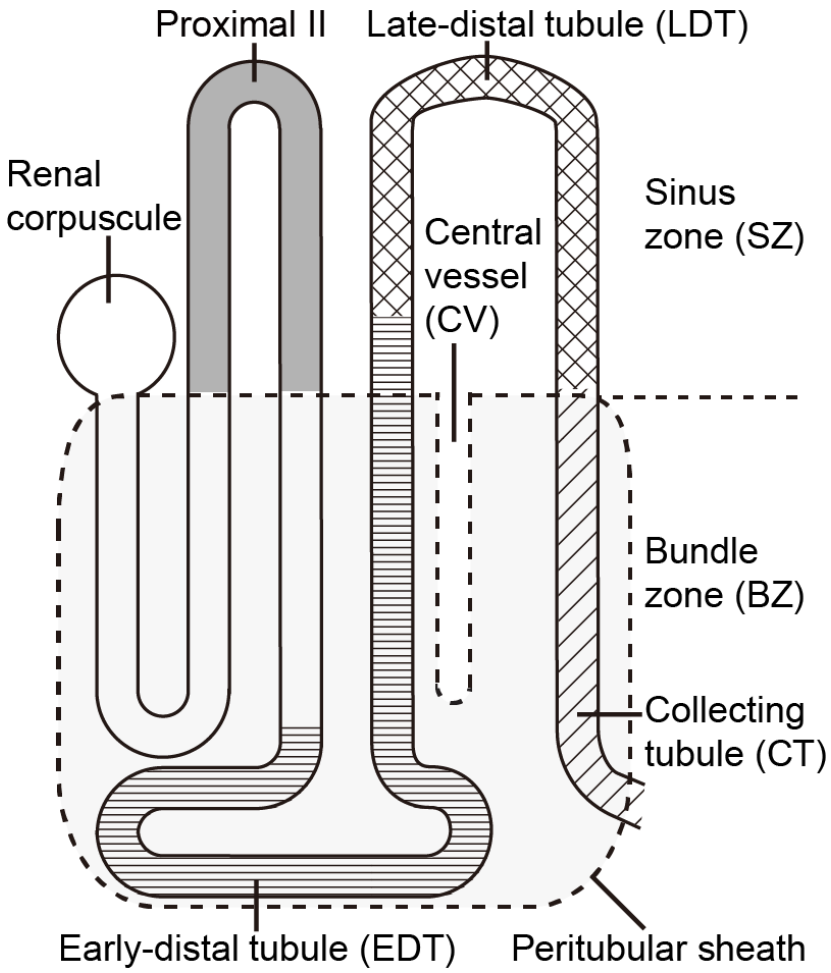

Fig. I Schematic representation of a typical single nephron in cartilaginous fish. The subdivisions of each tubular part are indicated by different patterns.

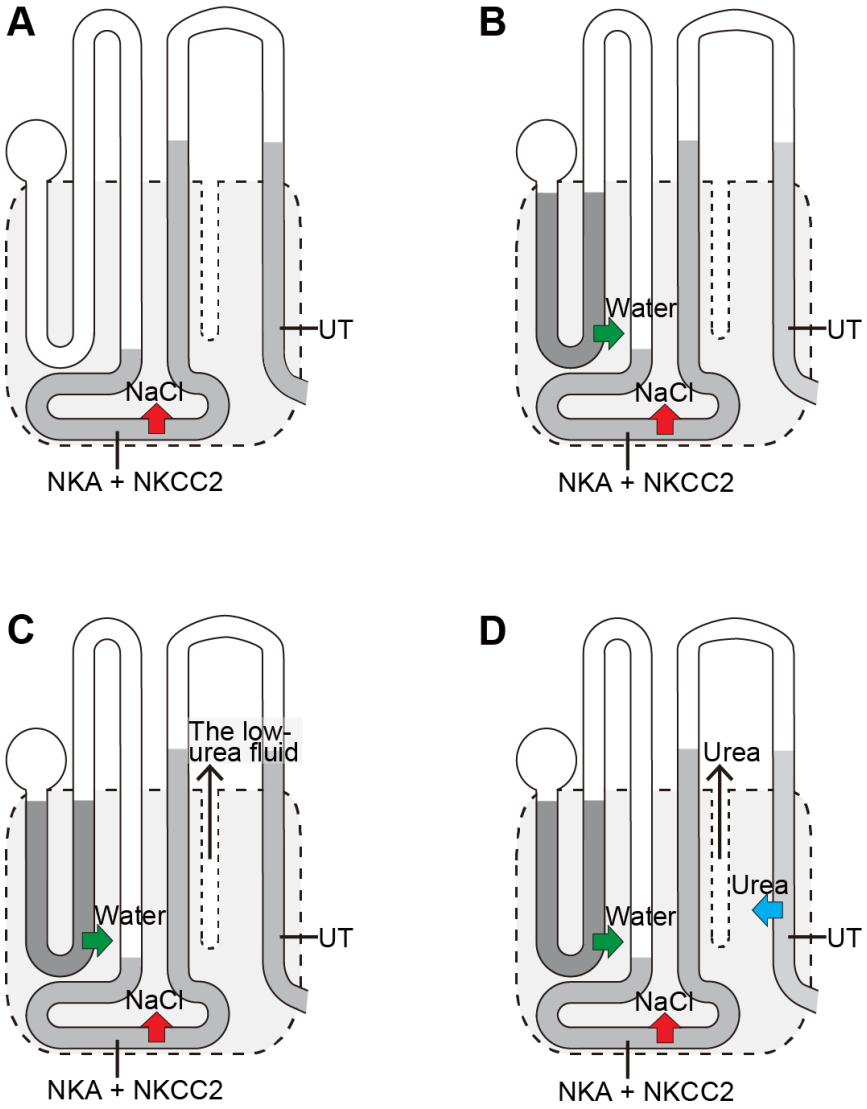

Fig. II Schematic diagram showing a urea reabsorption model in the kidney of cartilaginous fish. *A*: NaCl is reabsorbed from the EDT via Na^+/K^+-ATPase (NKA) and Na^+-K^+-Cl^- cotransporter-2 (NKCC2) (red arrow). *B*: Water is reabsorbed from the undefined segment(s) in the bundle zone (green arrow). *C*: Low-urea fluid flows unidirectionally in the central vessel to the renal portal system. Urea-rich forming urine passes into the collecting tubule. *D*: Reabsorption of urea occurs from the high-urea fluid in the collecting tubule to the low-urea environment in the central vessel via the urea transporter (UT) (blue arrow). Modified from Hyodo et al. (2014).

Chapter 1-1

Comprehensive analysis of genes contributing to euryhalinity in the bull shark, *Carcharhinus leucas* part 1: Na^+-Cl^- co-transporter is one of the key renal factors up-regulated in acclimation to low-salinity environment in bull shark, but not in houndshark, *Triakis scyllium*

Abstract

Most of the cartilaginous fishes live principally in seawater (SW), while limited species including the bull shark, *Carcharhinus leucas*, inhabit both SW and freshwater (FW) in their life cycles. Euryhaline elasmobranchs maintain high internal urea and ion levels even in low salinity environments, but little is known about the osmoregulatory mechanisms that enable them to maintain internal homeostasis in hypoosmotic environments. In the present study, the author focused on the kidney because this is the only organ that capable of excreting excess water from the body in a hypoosmotic environment. A transfer experiment of bull sharks from SW to FW and the subsequent differential gene expression analysis between the two conditions were conducted. A search for up-regulated genes in the kidney of FW-tranferred bull sharks indicated that the expression of the Na^+-Cl^- cotransporter (NCC; Slc12a3) was ten-folds higher in the FW-transferred group. In the kidney, apically-located NCC was observed in the late distal tubule and first-half of the collecting tubule where basolateral Na^+/K^+-ATPase was also expressed, suggesting that these segments contribute to NaCl reabsorption from the glomerular filtrate to produce diluted urine. The expression and localization of NCC are unique in bull shark since the kidney of houndshark, *Triakis scyllium* that had been transferred to 30% SW did not display similar features. The salinity transfer experiment combined with a comprehensive gene screening approach demonstrates that NCC is a key renal protein that contributes to the remarkable euryhaline ability of the bull shark.

Introduction

Most cartilaginous fishes are principally marine species, and only limited species, including bull shark *Carcharhinus leucas*, have the ability to inhabit both seawater (SW) and freshwater (FW) environments (see General Introduction; Compagno, 1984; Hazon et al., 2003; Pillans and Franklin, 2004). As described in General Introduction, marine cartilaginous fishes conduct urea-based osmoregulation for adaptation to high-salinity environment, and the kidney is important for urea retention. In SW kidney, more than 90% of filtered urea is reabsorbed from the primary urine to reduce the urea loss from urine. On the other hand, it was reported that FW-acclimated bull sharks still maintain high plasma osmolality (over 600 mOsm) with high plasma ion and urea concentrations (Pillans and Franclin, 2004; Pillans et al., 2006). Therefore, bull sharks must excrete excess water from osmotic influx from the hypoosmotic environments. Kidney is the only organ that can excrete excess water, thus it is highly possible that expression of key proteins is altered in response to the need of ion and urea reabsorption when producing diluted urine.

In the present study, a transfer experiment of captive bull sharks from SW to FW and a subsequent comprehensive search for transcripts exhibiting significant increases in expression levels in FW-acclimated bull sharks were conducted by RNA-sequencing (RNA-seq). Based on the differential gene expression profiles, quantitative PCR and *in situ* hybridization analyses were conducted, and they were revealed that the expression of Na^+-Cl^- cotransporter (NCC) was substantially upregulated in the late distal tubule (the 4th loop) and the collecting tubule (the final segment) by transfer of bull sharks from SW to FW. In contrast, houndsharks *Triakis scyllium* acclimated to 30% SW did not display the specific changes in renal NCC as observed in bull sharks. These findings indicate that the activation of renal NCC is associated to NaCl reabsorption associated, which is one of the crucial mechanisms for the euryhalinity of bull

shark.

Materials and methods

Animals

The bull sharks, *Carcharhinus leucas*, used in this study [n = 7, total length (TL) = 199.9 ± 16.0 cm, body weight (BW) = 94.1 ± 20.4 kg] were caught by set nets or reared in the Okinawa Churaumi Aquarium. They were kept in holding tanks filled with SW (20–23°C) in the aquarium under a constant photoperiod (12 h:12 h, L:D) and fed chopped fish. During the experimental period, they were kept without feeding.

Japanese banded houndsharks, *Triakis scyllium* (n = 9, TL = 68.1 ± 1.0 cm, BW = 1.0 ± 0.05 kg) were collected in Koajiro Bay, near the Misaki Marine Biological Station of the University of Tokyo. They were transported to the Atmosphere and Ocean Research Institute of the University of Tokyo and kept in 2×10^3 L holding tanks (20-22°C) under a constant photoperiod (12 h:12 h, L:D). The fish were fed on chopped squid for at least 2 weeks before the experiments and were kept without feeding during the experimental period (see Yamaguchi et al., 2009).

All animal experiments were conducted according to the Guidelines for Care and Use of Animals approved by the committees of the University of Tokyo and the Okinawa Churaumi Aquarium.

Transfer experiments

In March of 2012, three bull sharks kept in holding tanks were transferred to an experimental tank (15×10^3 L, filled with full-strength SW, 35 ppt) 3 days before the onset of salinity change. It was ascertained that they could swim comfortably in the new tank. On day 1 of the experiment, the salinity was adjusted by adding FW to achieve a salinity of 80% SW. On day 2, the same degree of salinity change was performed to produce 60% SW. On days 3-8, the

salinity of the tank was reduced by 10% per day, respectively, and the salinity was deemed to have reached nearly FW (less than 2 ppt) on day 8 (Fig. 1). Fishes were sacrificed on days 8-10. Two SW control fishes were maintained in the holding tank with running SW, and were sacrificed on days 8 and 9, and two further SW fishes were sacrificed in November 2011 and February 2013, respectively. For sampling, bull sharks were initially anesthetized with an intramuscular injection of midazolam (2.5 mg kg^{-1} BW) and then euthanized with an intravenous injection of 2,6-diisoprophylphenol (0.6-1.6 mg kg^{-1} BW). Blood samples were collected from the dorsal vasculature by a syringe and were centrifuged at 10,000 × *g* at 4°C for 10 min to obtain the plasma. Urine samples were collected using a urethral catheter that was inserted under anesthesia (All Silicone Foley Balloon Catheter, Create Medic Co. Ltd., Kanagawa, Japan). Plasma and urine samples were stored at -80°C until analysis. Ion and urea concentrations and the osmolality of plasma and urine were measured by Rapid Lab 1265 (Siemens Healthineers, Erlangen, Germany). Kidneys were dissected out and separated into the left and right halves. One half was frozen quickly in liquid nitrogen, while the other half was trimmed transversely to 1 cm slices and fixed in the Bouin's solution without acetic acid (saturated picric acid: formalin = 3:1) or 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) containing 150 mM NaCl and 350 mM urea at 4°C for 2 days. Other tissues (hypothalamus, pituitary, gill, ventricle, atrium, liver, muscle, stomach, intestine, rectum, and rectal gland) were also collected and were frozen or fixed as described above.

Frozen and fixed tissues of houndsharks were collected in the previous transfer experiment in 2009. In brief, houndsharks were kept in two tanks (1-2 × 10^3 L, filled with full-strength SW, 34 ppt). On day 1, salinity of one tank was adjusted to a salinity of 80% SW by adding FW. On days 2 and 3, the same degree of salinity change was performed to produce 40% SW. FW was added on day 4 to produce a final salinity of 30% SW (10 ppt, n = 4). Control fishes (n = 5)

were kept in full-strength SW during the dilution protocol. Fishes were maintained for 1 week in each salinity and then euthanized and sampled. Sampling procedures were as described in detail previously (Yamaguchi et al., 2009).

RNA-seq analysis

Total RNA was extracted from the frozen kidneys of SW (fish No. 2 and No. 3) and FW-acclimated (fish No. 5 and No. 6) bull sharks using the guanidium thiocyanate-phenol-chloroform mixture (ISOGEN, Nippon Gene, Toyama, Japan). The concentration and quality of the extracted RNA were assessed using a Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Waltham MA, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA), respectively. Non-stranded libraries were then constructed using a TruSeq total RNA sample preparation kit (Illumina, San Diego, CA, USA). Sequencing was performed on a HiSeq 1500 (Illumina) to obtain 127 bp pair-end reads, as documented previously (Tatsumi et al., 2015). The adaptor sequences and low-quality reads were discarded by Trim Galore! v0.3.1 (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) and a fastq quality filter v0.10.0 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The above-mentioned RNA-sequencing and the subsequent bioinformatics were conducted in collaboration with Dr. Kuraku and his laboratory members.

The trimmed reads were subjected to *de novo* assembly to obtain transcript contigs for each fish by Trinity version r20140717 (Grabherr et al., 2011). The contigs from each individual fish were then clustered by CD-HIT EST v4.6.1 (Fu et al., 2012) and TGICL v0.0.1 (Pertea et al., 2003) to produce the consensus contigs. The read trimming, transcriptome assembly, and merging contigs were performed according to a previous study (Hara et al., 2015). From these consensus contigs, open reading frames (ORFs) and the deduced amino acid sequences were

predicted by Transdecorder version r20140704 (Haas et al., 2013). The contigs were annotated by Trinotate version r20150708 (Bryant et al., 2017), which provides the homology information of known proteins and functional domains, as well as *ab initio* prediction of transmembrane and signal peptides. The trimmed sequence reads were then mapped to the contigs by bowtie2 v2.1.0 (Langmead and Salzberg, 2012) and the expressions were quantified by eXpress v1.5.1 (Cappé and Moulines, 2009). The differential expression analysis between SW and FW individuals was performed by edgeR v3.0.0 (Robinson et al., 2010; McCathy et al., 2012). The sequencing information and assembly statistics are shown in Table 1. The accession number for the short-read data of RNA-seq analysis is DRA008155.

cDNA cloning

Complementary DNA cloning was performed as previously described in detail (Hasegawa et al., 2016). Total RNA was extracted from the kidney with ISOGEN. 2 μg of total RNA was treated with DNase using a TURBO DNA-free kit (Life Technologies, Carlsbad, CA, USA) and reverse-transcribed to first-strand cDNA using a high-capacity cDNA reverse transcription kit (Life Technologies), following the manufacturer's instructions. Primers for cDNA cloning were designed to amplify entire ORFs based on the contig sequence data from the RNA-seq (Table 2). The target cDNAs were amplified with Kapa Taq Extra DNA polymerase (Kapa Biosystems, Boston, MA, USA), electrophoresed on a 1.2% agarose gel, excised and purified using UltraClean 15 DNA Purification Kit (MO BIO Laboratories, Carlsbad, CA, USA). The purified fragments were ligated into a pGEM T-easy vector (Promega, Madison, WI, USA), and the nucleotide sequence was determined by an automated DNA sequencer (3130xl Genetic Analyzer; Life Technologies). The accession numbers for the partial sequences of bull shark Na^+/K^+-ATPase alpha subunit 1 (NKAα1), Na^+-K^+-Cl^- cotransporter-2 (NKCC2), Na^+-Cl^-

cotransporter (NCC), urea transporter (UT), and elongation factor 1 alpha subunit 1 (EF1α1) are LC462277, LC462278, AB769491, LC462279 and LC462280, respectively. The accession numbers for the partial sequences of houndshark NKAα1, NKCC2, NCC, UT and EF1α1 mRNAs are AB669491, AB769486, AB769487, AB094993 and LC462281, respectively.

Quantitative real-time PCR assay

The expression levels of mRNAs were examined by real-time quantitative PCR (qPCR) method using a 7900HT Sequence Detection System (Applied Biosystems, Waltham, MA, USA) with KAPA SYBR Fast qPCR kit (Kapa Biosystems), as previously described in detail (Hasegawa et al., 2016). The plasmids containing cloned cDNAs were serially diluted and were used as the known amounts of standard cDNAs for absolute quantification in qPCR analyses. The numbers of molecules of the standard cDNAs were calculated using BioMath Calculators (http://www.promega.com/techserv/tools/biomath/calc01.htm). Primer sets for qPCR were designed using Primer Express software or PrimerQuest (http://sg.idtdna.com/primerquest/home/index) (Table 2). Expression of EF1α1 was used as an internal control. First-strand cDNAs were prepared from 11 tissues (hypothalamus, pituitary, gill, liver, muscle, ventricle, atrium, stomach, intestine, rectum, and rectal gland) as described above, and were used for a tissue distribution analysis.

***In situ* hybridization and morphological observation**

Kidneys fixed in 4% paraformaldehyde solution were embedded in Paraplast (McCormick Scientific, Richmond, IL, USA). Serial sections were cut at 7 μm thickness and mounted onto MAS-coated slides (Matsunami Glass, Osaka, Japan). The cDNA fragments of bull shark NKAα1 (765 bp), NKCC2 (845 bp), NCC (1079 bp) and UT (664 bp), and houndshark NKAα1

(859 bp), NKCC2 (936 bp), NCC (1080 bp) and UT (972 bp) were amplified using gene-specific primers (Table 2), and the purified PCR products were used as the templates to synthesize digoxigenin (DIG)-labeled antisense and sense cRNA probes (DIG RNA labeling kit; Roche Applied Science, Mannheim, Germany), following the manufacturer's protocols. Hybridization and washing were conducted using a previously described protocol (Takabe et al., 2012). Stained sections were counterstained with Nuclear Fast Red (Vector Laboratories, Burlingame, CA, USA). Micrographs were obtained using a virtual slide system (Toco; Claro, Aomori, Japan).

For morphological observations, kidney sections fixed in Bouin's solution were stained with periodic acid-Schiff (PAS; McManus, 1946). Briefly, deparaffinized sections were oxidized in 0.5 % periodic acid solution (Wako Pure Chemical Industries, Osaka, Japan) for 5 min. After washing in tap water and distilled water, sections were placed in Schiff's reagent (Wako) for 15 min, and then rinsed in sulfurous acid three times. Sections were then counterstained with Mayer's hematoxylin (Wako).

Immunohistochemistry

A peptide C+GFEDEAIVKELRKD from bull shark NCC was synthesized and coupled via cysteine to keyhole limpet hemocyanin. The conjugated peptide was emulsified with complete Freund's adjuvant and injected into a rat for immunization (Eurofins Genomics, Tokyo, Japan).

Kidneys fixed with Bouin's solution without acetic acid were embedded in Paraplast. Serial sections were cut at 7 μm thickness and mounted onto MAS-coated slides (Matsunami Glass). Rehydrated tissue sections were treated with antigen activator (Histo VT One; Nacalai Tesque, Kyoto, Japan) for 20 min at 90°C. Immunohistochemical staining for NCC was

performed with the avidin-biotin-peroxidase complex kit (Vector Laboratories) described previously (Hasegawa et al., 2016). NCC antiserum was diluted with 2% normal goat serum in PBS (pH 7.4) (1:90,000). The specificity of immunoreactive signals for NCC was confirmed by competitive displacement of antibody with the synthetic antigen for 24 h at 4°C prior to incubation. Stained sections were counterstained with Mayer's hematoxylin (Wako). Micrographs were obtained using a digital camera (DSRi1; Nikon, Tokyo, Japan). For double-labelling fluorescence immunohistochemistry, the NCC antiserum (1:30,000) and anti-NKAα subunit antibody (1:2,000; immunized in a rabbit; a gift from Prof. Kaneko) were mixed and incubated with deparaffinized sections for 48 h at 4°C. Sections were washed in PBS, and then incubated with fluorescein-labeled anti-rat IgG antibody (Alexa Fluor 555) and anti-rabbit IgG antibody (Alexa Fluor 488) (Thermo Fisher Scientific) for 2 h at room temperature. Sections were mounted with ProLong Gold Antifade Reagent (Thermo Fisher Scientific). Micrographs were obtained using a fluorescence microscope (BX53; Olympus, Tokyo, Japan).

Statistical analysis

Values are presented as means $\pm$ s.e.m. and were compared using Student's *t* test or the Mann-Whitney *U* test, with the assumption of normality checked by the Shapiro-Wilk test. *P*-values < 0.05 were considered statistically significant. All analyses were performed using KyPlot 5.0 software (Kyenslab, Tokyo, Japan).

Results

Changes in plasma and urine parameters after transfer to low-salinity environments

The composition of plasma and urine in SW- and FW-bull sharks are summarized in Table 3. Plasma osmolality, ion and urea concentrations were decreased after the transfer to FW, and the decrease in osmolality, Cl^- and urea were statistically significant. However, as previously reported (Pillans and Franklin, 2004), bull shark maintained plasma osmolality at approximately 600 mOsm/kg in the FW environment. Although the time course changes of plasma osmolality was not available, I can estimate that the osmolality of environmental water was lower than the plasma osmolality by day 2, while plasma Na^+ level was hyperosmotic to the external media by day 4 of the transfer experiment (Fig. 1). Therefore, the bull sharks were exposed to hypo-osmotic conditions for at least one week before sampling. Urine parameters, except urea, were also decreased in the FW group (Table 3).

In the present study, glomerular filtration rate (GFR) and urine flow rate (UFR) were not able to measure in SW- and FW-acclimated bull sharks. In Atlantic stingray, the transfer from ambient SW to 50% SW for 2 hours increased GFR and UFR 3-fold and 9-fold, respectively (Janech et al., 2006). In Table 4, the values for GFR and UFR reported in Atlantic stingray were used to estimate the quantity of reabsorbed NaCl in the bull shark kidney. The stingray GFR and UFR values in SW and 50% SW were used in the calculation of renal performance of SW- and FW-acclimated bull sharks respectively. The estimated values in Table 4 indicate that the FW transfer of bull sharks increased tubular NaCl reabsorption from the glomerular filtrate by approximately 50%.

The plasma parameters of houndsharks after transfer to 30% SW (10 ppt) were reported previously (Yamaguchi et al., 2009). Plasma osmolality and the concentration of urea and ions were decreased in the 30% SW group. The preliminary experiment showed that Japanese

banded houndshark cannot survive when the environmental salinity was lowered than 25% of SW (8 ppt) (see Yamaguchi et al., 2009).

Expression profiles of Slc protein-family mRNAs in the bull shark kidney

Renal mRNA expression profiles were examined in two SW and two FW bull sharks by RNA-seq to search for candidate genes exhibiting a significant difference in expression levels following the FW acclimation (see Materials and Methods). The clustering of the overall expression profiles showed a clear distinction between the two SW individuals and the two FW-acclimated individuals (Fig. 2), indicating that acclimation to the FW environment triggered changes in renal function at molecular level. The application of dual cutoffs [false discovery rate (FDR) < 0.05 and log 2-fold change (LogFC) > 1.5 of the Fragment Per kilobase of exon per Million (FPKM)] identified 138 genes upregulated in the FW-acclimated group. Among them, six genes encoding the solute carrier (Slc) family proteins were found to be upregulated: $Na^+/K^+/Ca^{2+}$ exchanger 5 (Slc24a5), carnitine transporter 2 (Slc22a5, OCNT2), Na^+-coupled neutral amino acid transporter 8 (Slc38a8), Ca^{2+}-binding mitochondrial carrier protein 2B (Slc25a25), Na^+-Cl^- cotransporter (NCC, Slc12a3), and glucose transporter 12 (Slc2a12) (Table 5). The Slc family is comprised of proteins that are responsible for osmolyte transport (Hediger et al., 2004). In this analysis, NCC showed the largest FPKM values in FW individuals (over 120 in both individuals) that were approximately 8-fold higher than those of SW individuals. Therefore, the author focused on NCC as a candidate gene for FW-acclimation in bull sharks in this study.

Tissue distribution of NCC mRNA in bull shark

Tissue distribution of NCC mRNA was examined with quantitative RT-PCR in four bull

sharks kept in SW and three bull sharks acclimated in FW (Fig. 3). Expression of NCC was predominant in the kidney. Expression of NCC was significantly higher in the kidneys of FW-individuals (Figs. 3 and 4). Minute expression was detected in the hypothalamus, pituitary, and gills, but no significant change was observed between bull sharks in SW and FW (Fig. 3).

Changes in gene expression of NKAα1, NKCC2, NCC and UT in the kidneys of bull sharks and houndsharks

To validate the results of the RNA-seq and examine the possible involvement of other genes, quantitative RT-PCR was performed for some selected genes. As mentioned above, the expression of NCC mRNA was 10-fold higher in the kidneys of FW bull sharks compared to that of SW individuals (Fig. 4). In addition to NCC, the author examined the mRNA levels of Na^+/K^+-ATPase alpha subunit 1 (NKAα1; Atp1a1; a driving force of NaCl reabsorption), Na^+-K^+-$2Cl^-$ cotransporter 2 (NKCC2; Slc12a1; a protein important for NaCl reabsorption in the mammalian thick ascending limb), and urea transporter (UT; Slc14a2) in the kidneys of bull sharks, based on previous knowledge in osmoregulation. The expression of NKAα1 was significantly higher in the kidneys of FW- than SW-individuals. In contrast, no significant changes among the expression of NKCC2 and UT was observed following the FW transfer. However, the mean values of UT expression in FW was 1.5-fold to that in SW (Fig. 4).

On the other hand, in the kidneys of houndsharks, no significant change was observed in the NKAα1, NKCC2, NCC and UT mRNA levels after transfer to diluted SW (30% SW) (Fig. 4). Furthermore, the NCC mRNA levels (NCC mRNA/EF1α1 mRNA) of houndsharks in SW were approximately 40 times lower than that of bull sharks in SW. If the FW-acclimated bull sharks are compared to houndsharks in 30% SW, the NCC expression is approximately 500 times higher in the former.

Distribution of mRNAs encoding NKAα1, NKCC2, NCC and UT in the kidney of bull sharks

Structurally, the kidney nephron organization of bull shark is similar to those of other marine cartilaginous fishes (Lacy and Reale, 1985; Hentschel et al., 1998; Hyodo et al., 2004a, Kakumura et al., 2015). Multiple lobules are present and each lobule is separated into two morphologically distinctive regions: a sinus zone and a bundle zone (Fig. 5A). The sinus zone occupied a large area in lobules and was filled with blood sinuses. Two types of tubules are present in sinus zone. Proximal segment II is characterized by larger tubule size and diameter and presence of apical brush border, which correspond to the 2nd loop of the nephron (open arrows in Fig. 5B). The late distal tubules (LDT, 4th loop, filled arrows in Fig. 5B) were characterized by smaller diameter and shorter epithelial cell height, and without apical brush-border. In contrast, renal tubules in the bundle zone were densely packed, and five tubular segments from a single nephron (the descending and ascending limbs of the first and the third loops, and the final segment) were enclosed in a peritubular sheath (Fig. 5C and D).

Figure 6 shows mRNA signals of NKAα1, NKCC2, NCC and UT in the bull shark kidney. In SW individuals, signals for NKAα1 mRNA were observed in both the bundle and the sinus zones (Fig. 6A). From the high resolution images, NKAα1 mRNA signals were localized at LDT in the sinus zone (filled arrows in Fig. 6B) and early distal tubule (EDT) that is characterized by the largest tubule in the bundle zone (open arrowheads in Fig. 6C). Some tubules near renal corpuscles also expressed NKAα1 mRNA signals and these tubules are the transitional segments from the EDT to the LDT (open arrow in Fig. 6B) and from the LDT to the collecting tubule (CT) (filled arrowhead in Fig. 6B). Weak signals of NKAα1 mRNA were observed in the CT in the bundle zone (filled arrowhead in Fig. 6C). Combining the observations, NKAα1 expression is prominent from the EDT to the CT (Fig. 6a). In FW-bull

shark, similar distribution of NKAα1 mRNA was observed as in SW individuals. However, the mRNA signals were generally stronger in FW individuals, particularly in the LDT and descending limb of the 3rd loop (Figs. 6D-F, 6b). Signals for NKCC2 mRNA were observed almost exclusively in tubules of the bundle zone in both SW- and FW-acclimated bull sharks (Figs. 6G-L). In the bundle zone, intense signals of NKCC2 mRNA were detected in the EDT (open arrowheads in Figs. 6I and L). In the sinus zone, only a small number of tubules located in the vicinity of renal corpuscles were positive for NKCC2 mRNA (open arrows in Figs. 6H and K). Three-dimensional analysis by assembly of serial sections revealed that the NKCC2-positive tubules in the sinus zone are the transitional segment from the EDT to the LDT (Figs. 6c and d). No difference was observed among the distribution and signal intensity of NKCC2 mRNA between SW and FW fish.

Signals for NCC mRNA were weak in SW individuals (Figs. 6M-O). The signals were detected in the sinus zone, but not in the bundle zone. The morphological characteristics showed that the NCC-positive tubules are LDT (filled arrows in Fig. 6N). Meanwhile, in FW-acclimated individuals, strong signals for NCC mRNA were observed in the LDT (Figs. 6P and Q). In addition, intense signals were also detected in the first half of the collecting tubule (CT) in the bundle zone (filled arrowheads in Fig. 6R, and schematically in Fig. 6f). Signals for UT mRNA were detected almost exclusively in the CT in the bundle zone in both SW- and FW-acclimated bull sharks (Figs. 6S and V), and the signal intensity was higher in FW-acclimated bull sharks than in SW individuals (filled arrowheads in Figs. 6U and X). A small number of tubules located in the vicinity of renal corpuscles within the sinus zone are UT-positive, and these segments correspond to the transition from the LDT to the CT (filled arrowheads in Figs. 6T and W). The intensity of UT mRNA signals in this segment was also higher in FW-acclimated bull sharks (Figs. 6g and h).

Co-expression of NKAα1, NKCC2, NCC and UT mRNAs was examined using the serial sections of FW individuals (Fig. 7). In the sinus zone, NCC mRNA was co-expressed with NKAα1 mRNA in the LDT (filled arrows in Figs. 7A and E). The LDT did not express NKCC2 mRNA and UT mRNA (Figs. 7C and G). NKCC2 mRNA was moderately expressed in the transitional segment from the EDT to the LDT, where NKAα1 mRNA was also expressed (open arrows in Figs. 7A and C). In the bundle zone, NKCC2 mRNA was co-expressed with NKAα1 mRNA in the EDT (open arrowheads in Figs. 7B and D), while co-expression of NKAα1 mRNA, NCC mRNA, and UT mRNA was found in the CT (filled arrowheads in Figs. 7B, F and H).

Distribution of NKAα1, NKCC2, NCC and UT mRNAs in houndshark kidneys

In the kidneys of SW houndsharks, intense positive signals for NKAα1 mRNA were observed in the EDT in the bundle zone (Fig. 8A, open arrowheads in Fig. 8C). The LDT (4th loop of nephron) also NKAα1-positive (filled arrows in Fig. 8B). In the LDT, the transitional segment from the EDT to the LDT possess higher expression of NKAα1 (open arrows in Fig. 8A and B) but not in the rest of LDT (Fig. 8a). No apparent difference was observed in the localization and signal intensity of NKAα1 mRNA between SW individuals and 30% SW-acclimated individuals (Figs. 8A-F, 8a and b).

The expression of NKCC2 mRNA was similar to that of NKAα1 mRNA (Figs. 8G-L). Intense signals for NKCC2 mRNA were detected in the EDT in the bundle zone (open arrowheads in Figs. 8I and L), while weak or moderate levels of NKCC2 expression were found in the LDT (open and filled arrows in Figs. 8H and K). The localization and signal intensity of NKCC2 mRNA between 100% SW and 30% SW groups are similar (Figs. 8G-L, 8c and d).

NCC mRNA was expressed only in the transitional segment from the LDT to the CT (Figs.

8M and filled arrowheads in 8N, schematically shown in 8e). In 30% SW-acclimated houndsharks, signals for NCC mRNA were weaker than those in SW individuals (filled arrowheads in 8Q). UT mRNA was expressed in the CT (Figs. 8S and filled arrowheads in 8U). In the sinus zone, intense signals for UT mRNA were detected in the transitional segment from the LDT to the CT (filled arrowheads in Fig. 8T), and the signal intensity of the transitional segment was decreased 30% SW individuals (filled arrowheads in Fig. 8W), similar to the pattern of NCC mRNA signals.

Co-expression of the four mRNAs was examined using the serial sections of SW individuals (Fig. 9). NKCC2 mRNA was consistently expressed with NKAα1 mRNA in the EDT (open arrowheads in Figs. 9B and D) and the LDT (open arrows in Figs. 9A and C). In the sinus zone, NCC mRNA was co-expressed with UT mRNA in the transitional segment from the LDT to the CT, where NKAα1 mRNA was also expressed (filled arrows in Figs. 9A, E and G). In contrast to the results in bull sharks, the CT only expressed UT but not other transporters (filled arrowheads in Figs. 9B, D, F and H).

Localization of NCC protein in the LDT cells of bull sharks

The intracellular localization of bull shark NCC was examined using the antiserum raised against a synthetic polypeptide of bull shark NCC. Consistent with the results of *in situ* hybridization, immunoreactive signals of NCC were observed in the LDT in FW individuals (open arrows in Fig. 10A), but not in SW individuals (Fig. 10D). The NCC protein was localized to the apical membrane. The use of pre-immune serum (Fig. 10B) and competitive displacement of the anti-NCC serum with the synthetic NCC polypeptide (Fig. 10C) removed the NCC immunoreactive signals. Double fluorescence immunohistochemistry showed that NCC was localized on the apical membrane while NKA was localized on the basolateral membrane in the

same tubular cell of LDT (Fig. 10F).

Discussion

To understand the mechanisms of euryhalinity in bull sharks, the author focused on kidney function in the present study, since the kidney is the only organ that can excrete excess water in a hypoosmotic environment. By the combination of RNA-seq analysis and the subsequent molecular and histochemical analyses, it was found that NCC in the LDT and CT is one of the key transporters contributing to the successful FW acclimation in bull sharks.

Reabsorption of NaCl from the glomerular filtrate

In SW, bull sharks, like all other marine cartilaginous fishes, maintain their plasma slightly hyperosmotic to surrounding SW by accumulating urea in the body (see also Pillans and Franklin, 2004). Transfer to a low-salinity environment resulted in a decrease in plasma ion and urea levels. However, the FW-acclimated bull sharks still maintained high plasma osmolality (617.7 $\pm$ 13.0 mOsm) in the present study as well as previous reports (Pillans and Franklin, 2004; Pillans et al., 2006). This phenomenon is also true in other euryhaline cartilaginous fishes such as Atlantic stingray *Hypanus sabina* (Piermarini and Evans, 1998). The osmolality difference between body fluid and external media suggests that bull shark experiences severe hypo-osmotic stress and must excrete a large volume of diluted urine to counter the osmotic influx from diluted media. In the present study, however, due to technical limitations, measurement of glomerular filtration rate (GFR) and urine flow rate (UFR) of SW- and FW-acclimated bull sharks were not conducted, and thus the water excretion and NaCl reabsorption were not able to quantify in the kidneys after FW transfer. Therefore, the values of GFR and UFR were extrapolated from the report in Atlantic stingray transferred from SW to 50%-diluted SW (Janech et al., 2006) to estimate the amount of reabsorbed NaCl in the bull shark kidney (Table 4). The estimated values in Table 4 indicate that the transfer of bull sharks from SW to

FW increased NaCl reabsorption from the glomerular filtrate by 50%.

It should be noted that, because of the larger osmolality difference between plasma and and external media, bull sharks in FW should receive a larger water influx than the case of Atlantic stingrays in 50% SW. In other words, bull sharks in FW likely filter more plasma and excrete more urine than stingrays. The 1.5 times increase in NaCl reabsorption in FW bull sharks is lower than the value (2.5 times) reported in stingrays in 50% SW (Janech et al., 2006). However, a greater increase in NaCl reabsorption is assumed in FW-acclimated bull sharks. Therefore, the author searched for up-regulated mRNAs encoding solute carrier family proteins involved in NaCl reabsorption.

NCC in the LDT: a key molecule contributing to euryhalinity of bull shark

The expression of NKAα1 and NKCC2 in the kidney has been investigated in another cartilaginous fish, elephant fish (Kakumura et al., 2015). NKCC2 (Slc12a1) is well-known to be localized in the thick ascending limb of mammalian Henle's loop together with NKA, where NaCl is actively reabsorbed for dilution of primary urine (Fenton and Knepper, 2007). In the elephant fish kidney, NKAα1 and NKCC2 were colocalized in the early distal tubule (EDT) and the second-half of late distal tubule (LDT) (Kakumura et al., 2015), and the NaCl uptake by NKCC2 is considered to be important for the urea reabsorption process (Hyodo et al., 2014). However, no significant change was observed in the distribution and expression levels of NKCC2 mRNA following the transfer of bull sharks to FW environment.

A remarkable increase of NCC expression was detected in RNA-seq analysis and confirmed by qPCR. Only a modest NCC mRNA signal was found in the LDT of bull shark kidney in SW, while NCC signals considerably increased in the LDT of FW-acclimated bull sharks. NCC is known to be expressed in the apical membrane of the mammalian distal

convoluted tubule (Fenton and Knepper, 2007) and in the distal segment of the teleost fish kidney (Kato et al., 2011; Teranishi et al., 2013). Apical localization of NCC in bull sharks was also confirmed by the immunohistochemistry results in the present study. In the LDT, NCC is co-expressed with NKAα1, which is the driving force for NaCl reabsorption via NCC (Fenton and Knepper, 2007). The upregulation of NCC and NKAα1 and their colocalization strongly suggest that the LDT contributes significantly to NaCl retention in FW. The NCC is an evolutionary selected transporter that allow bull shark to overcome the high osmotic stress in FW by enhancing the NaCl reabsorption in the LDT (Fig. 11).

In cartilaginous fishes, NCC mRNA expression was identified in the gills and kidney of Japanese houndshark *T. scyllium* (Takabe et al., 2016). The present qPCR analysis revealed that expression of NCC in houndshark kidney was considerably lower than that of FW-acclimated bull sharks. No significant change was observed between the NCC expression in SW- and 30% SW-houndsharks. *In situ* hybridization study further demonstrated that NCC mRNA was expressed only in the LDT of houndshark kidney. In contrast to the kidney, NCC mRNA levels and the number of NCC-expressing cells in the gill increased after the SW to 30% SW transfer (Takabe et al., 2016). In houndsharks, NCC is likely to be involved in the absorption of NaCl from the environment. Although the transfer procedures were different between the experiment of bull sharks and houndsharks, significant up-regulation of NCC mRNA expression in the LDT was unique to bull shark, further implying that the NaCl reabsorption in the LDT is critical for the euryhalinity of bull shark.

NCC in the collecting tubule (CT): NaCl reabsorption and/or urea reabsorption?

In addition to the LDT, upregulation of NCC mRNA expression was also found in the CT. Colocalization of NCC and NKAα1 mRNA was found in the CT, implying reabsorption of

NaCl continue from LDT to CT in FW bull sharks. On the other hand, neither NKAα1 nor NCC mRNA signals were detected in the CT of houndshark kidney. Therefore, the CT-specific NaCl reabsorption is another unique feature contributing to the euryhalinity of bull shark. The species-specific elaboration of tubular NaCl reabsorption may enhance the effectiveness of NaCl retention in hypoosmotic environments (Fig. 11).

In addition, NaCl reabsorption in the CT may be also important for urea retention. Based on the exclusive localization of UT in CT, the segment has been known for its urea reabsorbing role in cartilaginous fishes (Hyodo et al., 2004a; Yamaguchi et al., 2009; Hyodo et al., 2014; Kakumura et al., 2015). The specific expression of UT in the CT is also true in bull shark kidney, implying that urea reabsorption in CT is common in both marine and euryhaline cartilaginous fishes. A model for urea reabsorption was proposed (Fig. II) and first step of this model is massive reabsorption of NaCl to produce a low urea environment in the tubular bundle. The low concentration of urea is the driving force for the urea absorption in CT via UT (Hyodo et al., 2014). The enhanced NaCl uptake in the tubular bundle can increase water uptake to steepen the concentration gradient along the bundle, thus facilitate the urea reabsorption. The reabsorbed urea in the FW-acclimated bull shark kidney was approximately 2/3 of that in bull sharks in SW (Table 4), but the author considers that bull sharks in FW should have a greater GFR and UFR to excrete excess water. Under this scenario, bull sharks in FW reabsorb more urea than in the SW to maintain the high plasma urea levels (Fig.11).

Currently, the function of the LDT in SW environment remains to be clarified. NKCC2 is expressed in the LDT of houndsharks (Fig. 6) and elephant fishes (Kakumura et al., 2015), and the segment is considered to be important for concentrating urea for subsequent urea reabsorption in the CT (Hyodo et al., 2014). However, in the bull shark kidney in SW

environment, neither NKCC2 nor NCC was prominent in the LDT (Fig. 11). Further studies are needed to understand the function of LDT in the kidney of bull sharks in the SW environment, and thus the changes of other candidate genes were examined in the following chapters.

Table 1. The assembly statistics of RNA-sequencing

	Number of pairs (x 10^6)					CEGMA referring to CVG	
RNA source	Before QC	After QC	Number of contigs	Number of predicted genes*	N50 length (bp)	Complete (%)	Partial (%)
Kidney from SW No.2	14.2	13.8	151,720	128,754	1,207	71.24	83.69
Kidney from SW No.3	15.2	14.9	179,734	135,902	1,271	77.25	87.98
Kidney from FW No.5	16.3	14.3	189,356	161,324	1,201	81.12	90.56
Kidney from FW No.6	15.8	14.9	196,057	168,624	1,256	83.69	92.70
Merged	-	-	305,524	268,917	1,468	91.42	97.42

*Genes were defined as clusters of the contigs based on the sequence overlapping.

Note. QC: Quality control. CVG is referred from Hara et al., 2015.

Table 2. Primer sets used in the present study.

Gene name		Primer sequence 5' to 3'
For bull shark		
Primer sets for cDNA cloning and *in situ* hybridization		
NKAα1	Sense	TTGTGCAACCGTGCCGTC
	Antisense	AGATGTCGTCTAGCTGTTCT
NKCC2	Sense	GCACTTATCAACTTTTCATCT
	Antisense	TTTCAGTGAACGGATTCTTC
NCC	Sense	GGGTACGGGAAGAATAACGAGC
	Antisense	CTTTCTTTCCTCATCCACACGAT
UT	Sense	CAGAATCCATGGTGGGCACT
	Antisense	ACAGGACTGTACCGTCTGAG
EF1α1	Sense	AAACGATACGACGAAATCACAAA
	Antisense	CATCTCCAGACTTCACAAATTTTG
Primer sets for qPCR		
NKAα1	Sense	CTGTTGCCTGACGACACA
	Antisense	ACAAAGCATAGATCAATGAGG
NKCC2	Sense	CAGGCCACAGTGTATCGTAC
	Antisense	GTAAATACTTGACTACAGATGCAG
NCC	Sense	TGATTTGCGAAGTGGAACACA
	Antisense	ATCACAAGTACGTTGGGCTTCA
UT	Sense	TGGCTGCAACAGGACACAAT
	Antisense	TTGCAATGAAGTAACTGGTCCAA
EF1α1	Sense	AGACGGTAAGGTCACAGGACACA
	Antisense	GCTTATAGGGCGTTCTGGAGGTA
For houndshark		
Primer sets for standard cDNAs of qPCR		
NKAα1	Sense	CTCAAAGACCTAACCGCAGA
	Antisense	CCTCAGTGCTATATCAGTACCGG
NKCC2	Sense	CATCGGTATTGCTAACTTTTT
	Antisense	CTTGCCAAAGAAATACAGC
NCC	Sense	GTTGGCTTCCTTCTGGCT
	Antisense	CAACAGCACTGGAGTCCAC

UT	Sense	ACTGGCTGAAAGAGCAGAAC
	Antisense	AGCACTACAGGCCAAAACGC
	Antisense	CAGGGTAGGTCACTTCAGATAGA
EF1α1	Sense	AAACGATACGACGAAATCACAAA
	Antisense	CATCTCCAGACTTCACAAATTTTG
Primer sets for *in situ* hybridization		
NKAα1	Sense	GGTGCCATTGTAGCTGTGAC
	Antisense	TATAAGGGAAGGCGCAGAACCACCA
NKCC2	Sense	ACGGTTGCGGGGATGGAGTGGGA
	Antisense	TGCCCACCAGTTTATTACGAACAT
NCC	Sense	GGGTACGGGAAGAACAACGA
	Antisense	CTTACGCTCTTCGTCCATCC
UT	Sense	(Same as the cloning primer)
	Antisense	CAGGGTAGGTCACTTCAGATAGA
Primer sets for qPCR		
NKAα1	Sense	TGCTTACACTTTAACCAGCAATATCC
	Antisense	GGCTGTCTCTTCATTATATCACTTTC
NKCC2	Sense	ATCAATGATACTATAAGTTCTTCGCTGAA
	Antisense	TATGATTTCTCTACCTGTAATGCACAGG
NCC	Sense	GGGAACTGTCACCAAAAACCAC
	Antisense	CTGAGATATGCTCTAGTGGACACATCA
UT	Sense	TGGCTGCAACAGGACACAAT
	Antisense	TGCGATGAAGTAACTGGTCCAA
EF1α1	Sense	CCTCCAGAACGCCCTGTAAG
	Antisense	GTACCAATACCACCAATCTTGTAGACA

Table 3. Plasma and urine composition of bull sharks in SW and FW

Contents	Experiment group	*N*	Osmolality (mOsm kg^{-1})	Na^+ (mmol l^{-1})	Cl^- (mmol l^{-1})	Urea (mmol l^{-1})	K^+ (mmol l^{-1})	Ca^{2+} (mmol l^{-1})	Mg^{2+} (mmol l^{-1})
Plasma	Bull shark (SW)	4	1031.0 ± 24.9	290.3 ± 20.0	239.3 ± 17.0	355.1 ± 2.7	3.3 ± 0.1	4.7± 0.1	2.1 ± 0.1
	Bull shark (FW)	3	617.7 ± 13.0***	213.7 ± 9.0	177.0 ± 3.5*	144.4 ± 13.0**	3.0 ± 0.7	2.8 ± 0.1***	0.8 ± 0.1***
Urine	Bull shark (SW)	3	849.3 ± 100.5	282.7 ± 53.5	236.0 ± 14.5	105.9 ± 30.5	88.3 ± 25.7	10.2 ± 3.5	15.4 ± 5.6
	Bull shark (FW)	3	480.7 ± 43.0	162.7 ± 13.9	143.7 ± 10.8*	122.0 ± 4.7	7.43 ± 1.6	2.4 ± 0.3	1.2 ± 0.1
SW			1067.0	550.0	510.0	ND	11	16.2	35.2

Values are means ± SEM. ND, not detectable. Statistically significant differences between SW and FW are shown with asterisks. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Table 4. Hypothetical absolute amounts of solutes reabsorbed from the glomerular filtrate

Groups	*N*	Reabsorption rate of osmolytes (μmol h^{-1} kg^{-1})		
		Na^+	Cl^-	Urea
Bull shark (SW)	3	813.4 ± 124.0	665.4 ± 85.8	1256.4 ± 30.7
Bull shark (FW)	3	1331.9 ± 47.5*	1031.1 ± 68.8*	802.6 ± 190.4

Values are means ± SEM. For the calculation, the values of glomerular filtration rate and urine flow rate of Atlantic stingray were used from Janech et al., 2006. Statistical analysis was performed using *t*-test (Na^+ and Cl^-) or Mann-Whitney *U* test (urea). Statistically significant differences between SW and FW are shown with asterisks. *, $P < 0.05$ (Na^+, $P = 0.01748$; Cl^-, $P = 0.02922$).

Table 5. Differentially expressed Slc family genes identified in the transcriptome analysis

Transcript contig ID	Gene symbol	Putative gene product	FPKM value						FDR
			SW No.2	SW No.3	FW No.5	FW No.6	FW/ SW	Log FC	
c13993_g0	Slc24a5	$Na^+/K^+/Ca^{2+}$ exchanger 5	0	0.05	1.19	5.09	125.60	6.616	1.21×10^{-5}
c37168_g0	Slc22a5	Carnitine transporter 2	0.05	0.14	2.73	1.05	19.89	3.948	1.54×10^{-2}
c218567_g0	Slc38a8	Na^+-neutral amino acid transporter 8	0.04	0.17	1.29	3.05	20.67	3.806	1.33×10^{-2}
c8778_g0	Slc25a25	Ca^{2+}-binding mitochondrial carrier protein 2B	8.35	12.56	216.6	35.3	12.05	3.161	3.00×10^{-3}
c24229_g0	**Slc12a3**	**Na^+-Cl^- co-transporter (NCC)**	**12.73**	**19.55**	**125.48**	**124.87**	**7.76**	**2.596**	**3.52×10^{-5}**
c1052_g0	Slc2a12	Glucose transporter 12	16.91	25.75	156.77	94.23	5.88	2.269	1.44×10^{-3}
Other functionally associated genes specifically analyzed in this study for NCC.									
c791_g0	Slc12a1	Na^+-K^+-Cl^- cotransporter2 (NKCC2)	201.37	159.48	185.2	256.42	1.22		
c41982_g0	Slc14a2	Urea transporter (UT)	52.19	43.28	50.28	74.45	1.31		
c88436_g0	Atp1a1	Na^+/K^+ ATPase subunit α1 (NKAα1)	646.42	780.25	1000.47	867.94	1.31		

FPKM, Fragments Per Kilobase of exon per Million reads mapped. This list includes only the contigs annotated as solute carrier (Slc) family members that satisfy the condition applied [FDR (false discovery rate) < 0.05; LogFC (Log 2-fold change) > 1.5].

Table 6. The creatinine concentration and reabsorption rate

	Creatinin (mg/dl)			Reabsorption rate (%)		
	Scr	Ucr	Concentration rate	Na^+	Cl^-	Urea
No.2 SW	0.1	10.3	103	98.9	99.0	99.6
No.3 SW	0.1	25.3	253	99.8	99.7	99.9
No.9 SW	0.1	51.3	513	99.7	99.7	100.0
No.4 FW	0.1	0.1	1	17.7	10.4	2.6
No.5 FW	0.1	0.1	1	23.9	18.1	6.2
No.6 FW	0.1	0.6	6	88.4	88.0	88.8

Scr, Plasma creatinine concentration. Ucr, urine creatinine concentration. Concentration rate = Ucr / Scr. Reabsorption rate = 100 – 100 × Urine concentration / (Plasma concentration × Concentration rate).

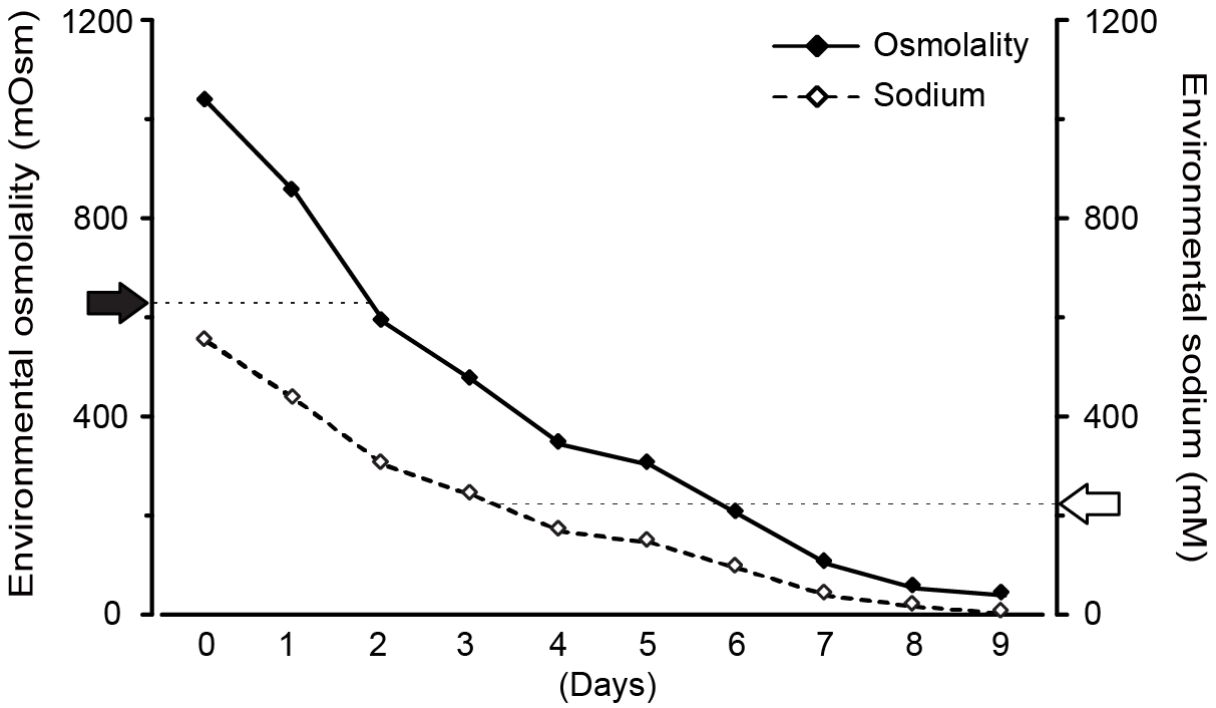

Fig. 1. Changes in osmolality and Na^+ concentration of the aquarium tank water during the FW-acclimation experiment. Filled circle and solid line show the osmolality of tank water and open circle and broken line show the Na^+ concentration of that. Note that osmolality and Na^+ concentration of the tank water fell below than the plasma values of FW-acclimated individuals after day 2 (filled arrow) and day 4 (open arrow), respectively.

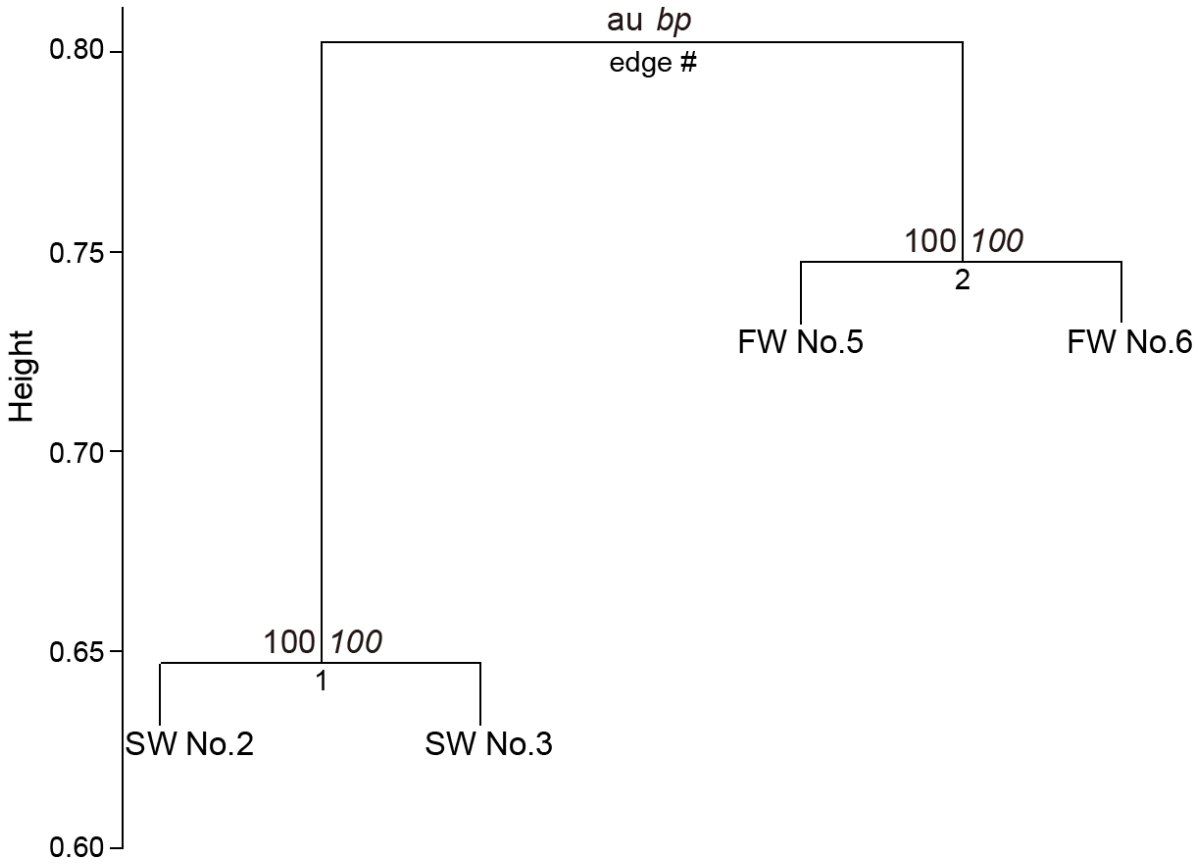

Fig. 2. Clustering analysis based on the expression profiles from transcriptome analysis. Two values of each node represent *P* values of approximately unbiased test (au) and bootstrapping probability (*bp, in italics*) for supporting the bifurcation. See the manual for Pvclust for details (https://cran.r-project.org/web/packages/pvclust/).

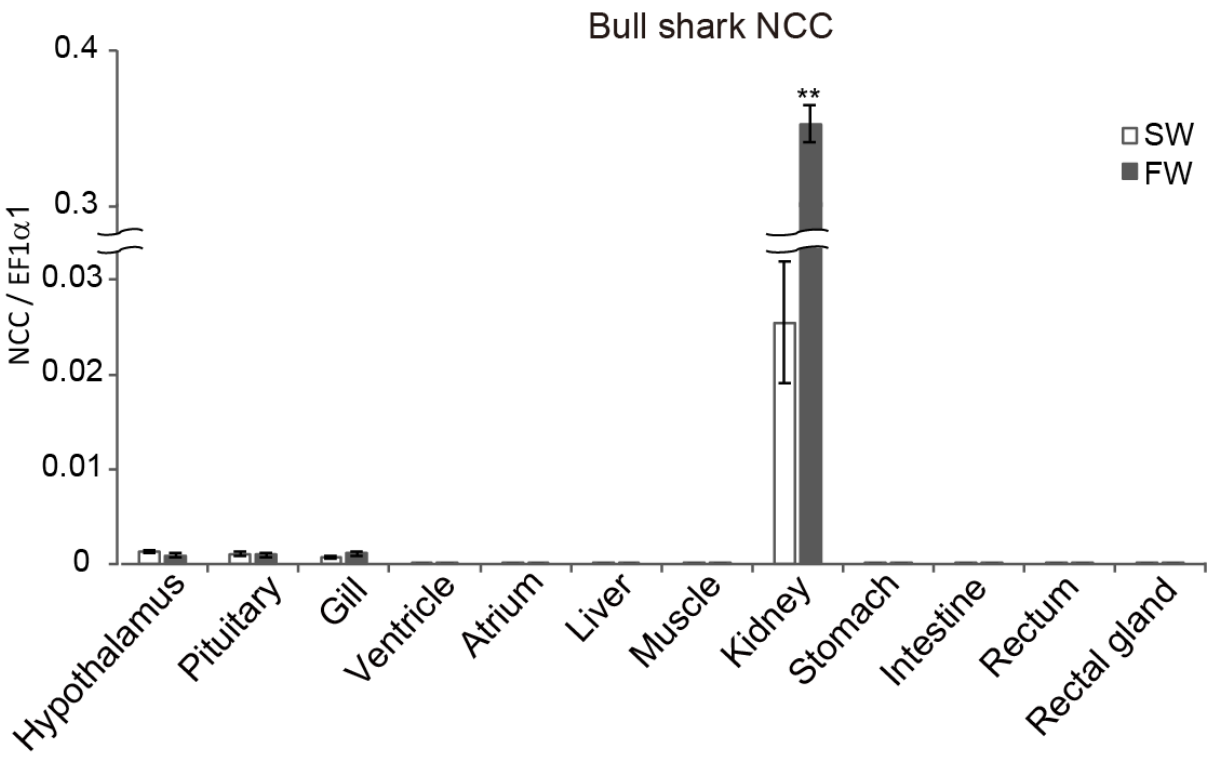

Fig. 3. Tissue distribution of NCC mRNA in the SW-acclimated (open bars) and FW-acclimated (filled bars) bull sharks. Data are expressed as the mean ± SEM. (SW, $n = 4$; FW, $n = 3$). Statistical analysis was performed using *t* test (except for rectal gland) or Mann-Whitney *U* test (rectal gland). Statistically significant differences are shown with asterisks. **, $P < 0.001$ (kidney, $P = 0.000004$).

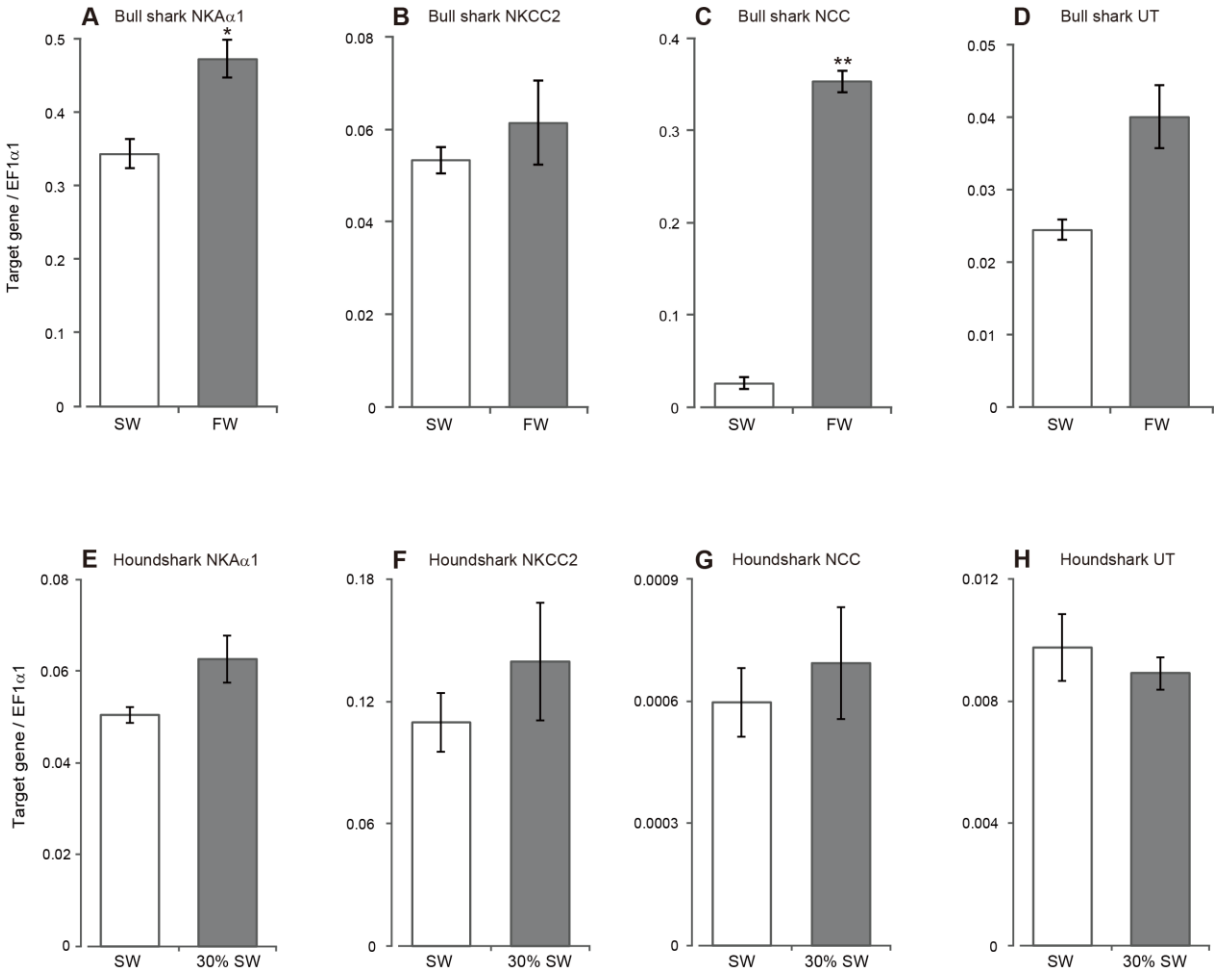

Fig. 4. Transcript quantification with real-time RT-PCR. The mRNA expression levels are shown for NKAα1 (A, E), NKCC2 (B, F), NCC (C, G) and UT (D, H) in the bull shark kidneys (A, B, C, D) and houndshark kidneys (E, F, G, H). Open bars represent SW (control) individuals, whereas filled bars show FW- or 30% SW-acclimated fish. Data are expressed as the mean ± SEM. $n = 4$ (SW bull sharks); $n = 3$ (FW bull sharks), $n = 5$ (SW houndsharks); $n = 4$ (30% SW houndsharks). Statistically significant differences (*t* test) are shown with asterisks. *, $P < 0.05$; **, $P < 0.001$ (bull shark NKAα1, $P = 0.0192$; bull shark NCC, $P = 0.000004$).

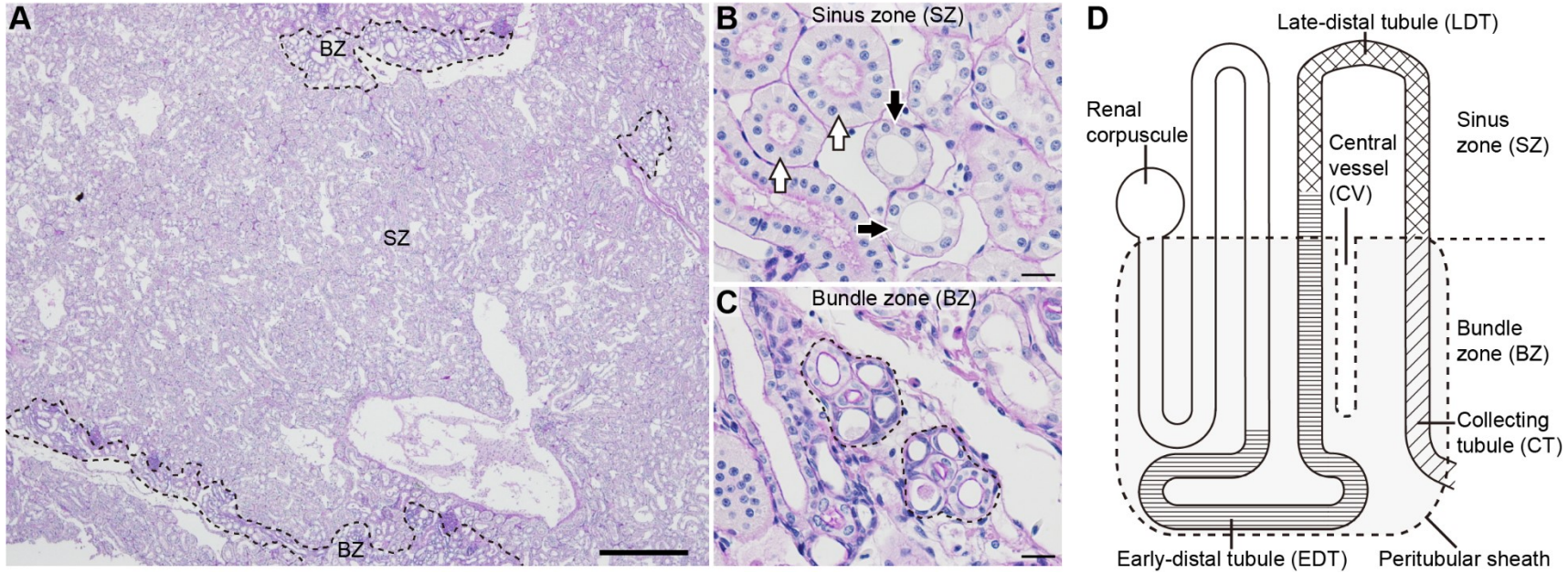

Fig. 5. Representative kidney sections showing the sinus zone (SZ) and the bundle zone (BZ) of the bull shark kidney. Sections were stained with hematoxylin and periodic acid-Schiff (PAS) (A-C). B and C are magnified views of SZ and BZ, respectively. Open arrows and filled arrows represent the 2nd loop and the LDT (4th loop), respectively. Bars = 500 μm (A) and 50 μm (B, C). D, schematic representation of the bull shark nephron.

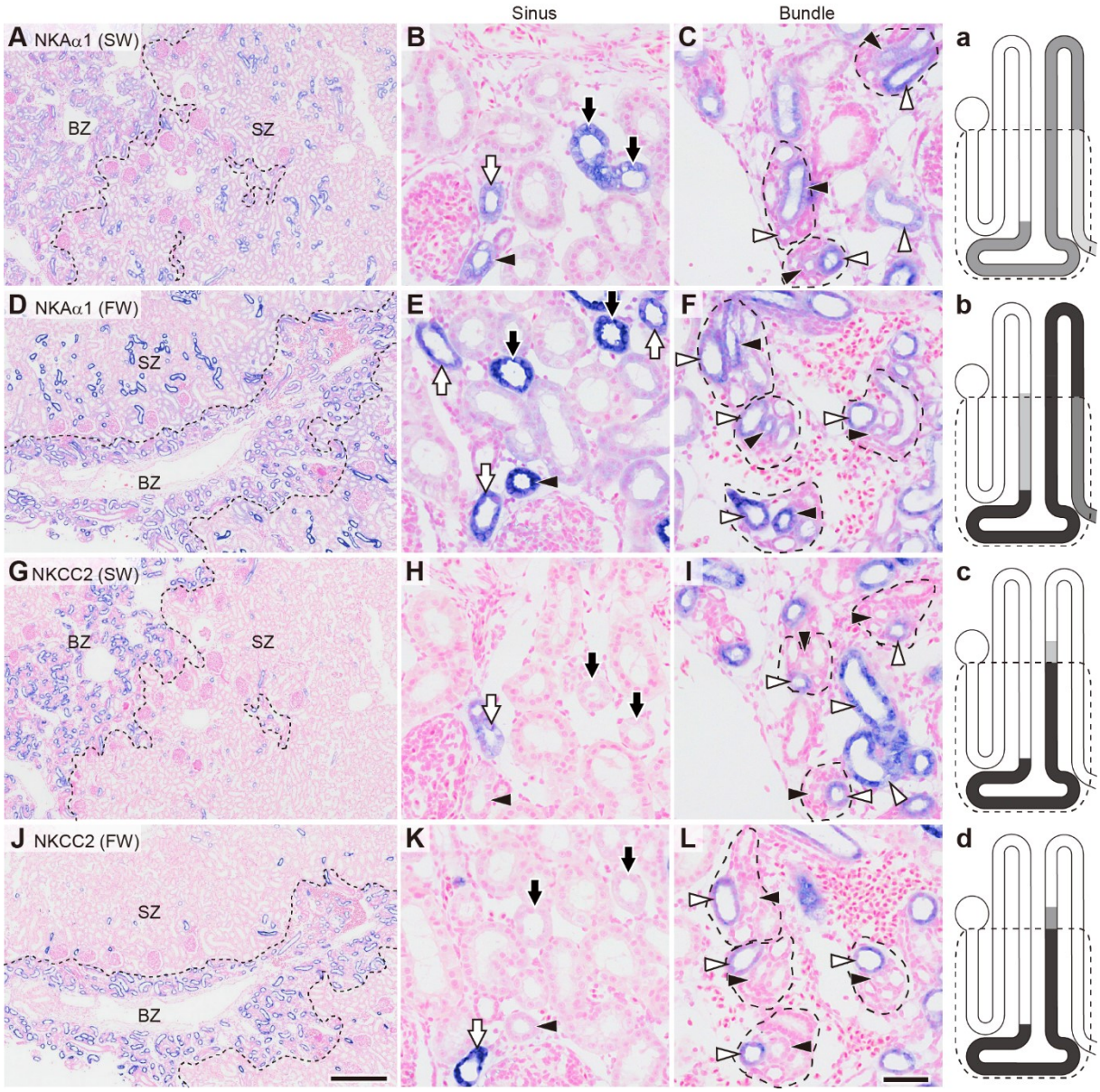

Fig. 6. *In situ* hybridization analyses of mRNAs encoding NKAα1, NKCC2, NCC and UT in the kidneys of bull sharks acclimated to SW and FW. NKAα1 (A-F), NKCC2 (G-L), NCC (M-R) and UT (S-X) mRNAs in SW- (A-C, G-I, M-O, S-U) and FW- (D-F, J-L, P-R, V-X) acclimated bull sharks. B, E, H, K, N, Q, T, W and C, F, I, L, O, R, U, X are magnified views of sinus zone (SZ) and bundle zone (BZ), respectively. The localization of each mRNA in the nephron is schematically illustrated (a-h). Open arrows, filled arrows, and open arrowheads represent the transitional segment from the EDT to the LDT, the LDT, and the EDT, respectively.

Filled arrowheads represent the transition segment from the LDT to the CT in the sinus zone, and the CT in the bundle zone. Note that NCC mRNA was intensely expressed in the LDT and the anterior part of CT in FW-acclimated bull shark kidney. Sections were counterstained with Nuclear Fast Red. Bar = 400 μm (A, D, G, J, M, P, S and V) and 50 μm (B, C, E, F, H, I, K, L, N, O, Q, R, T, U, W and X).

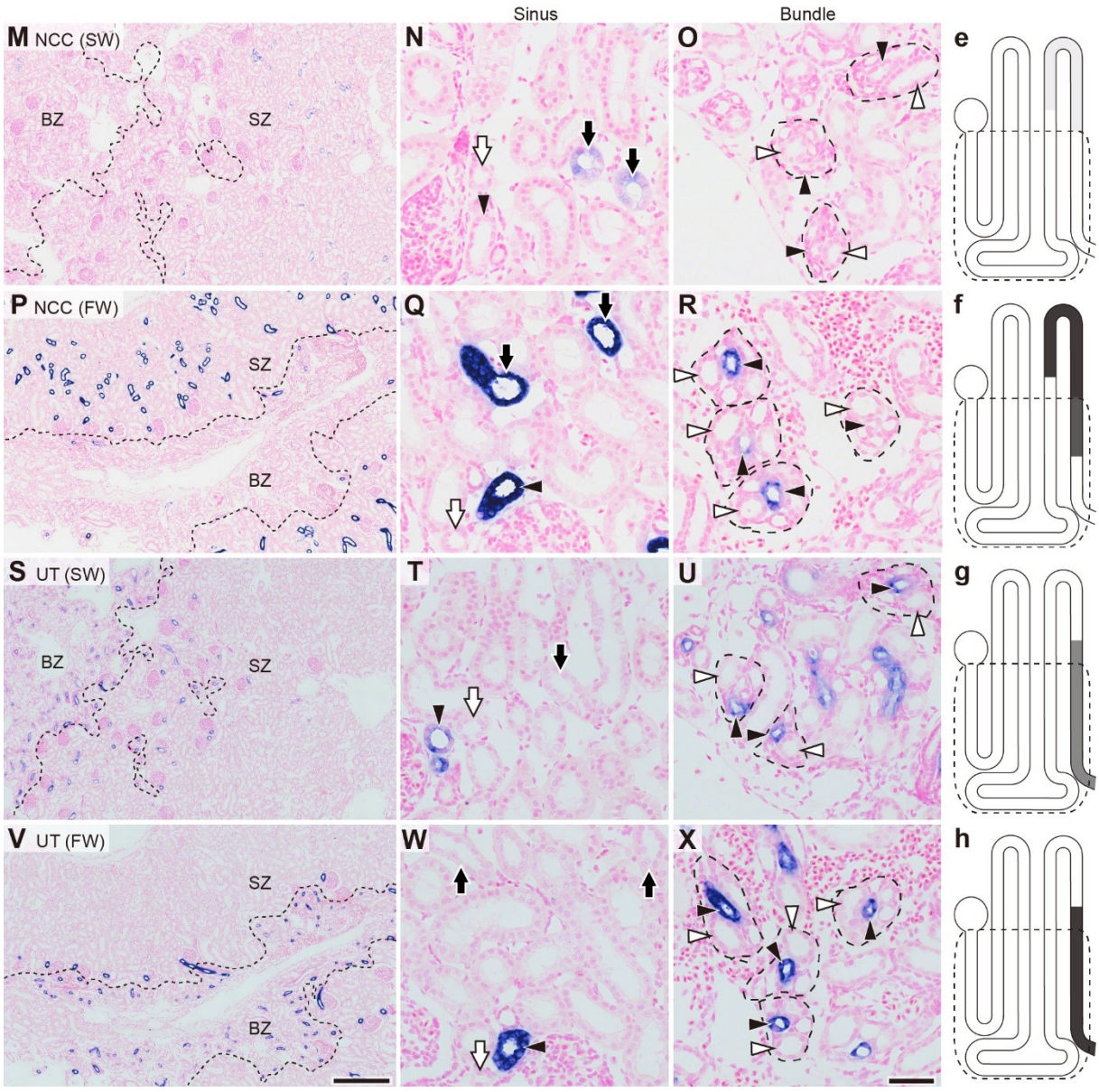

Fig. 6. Continued.

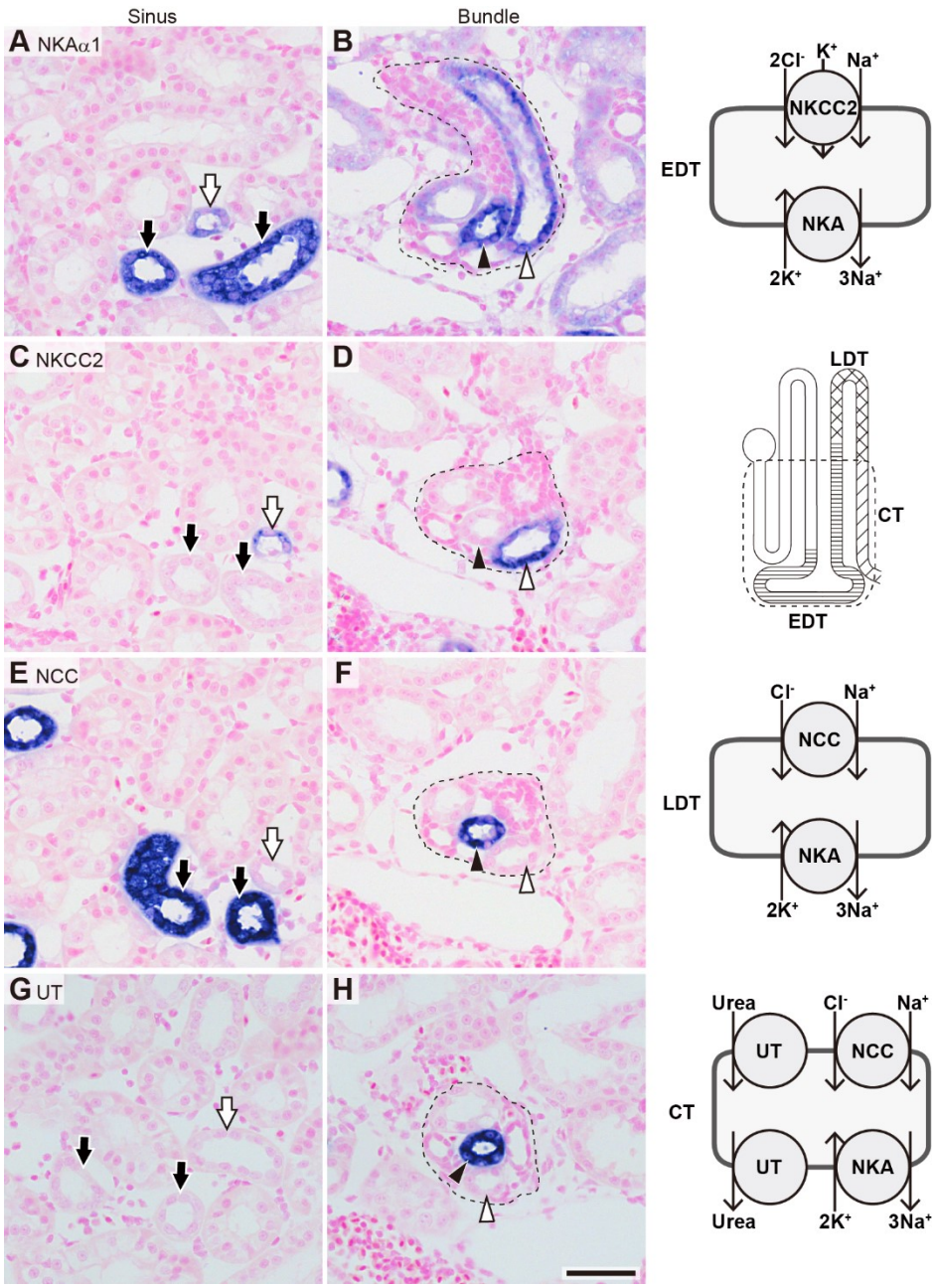

Fig. 7. Co-localization of mRNA signals in the kidney of bull shark in FW. NKAα1 (A, B), NKCC2 (C, D), NCC (E, F) and UT (G, H) mRNAs in the sinus zone (A, C, E, G) and bundle zone (B, D, F, H). Filled arrows and open arrows in the sinus zone represent the LDT and the transitional segment from the EDT to the LDT, respectively. Filled arrowheads and open arrowheads in the bundle zone represent the CT and the EDT, respectively. Sections were counterstained with Nuclear Fast Red. Bar = 50 μm.

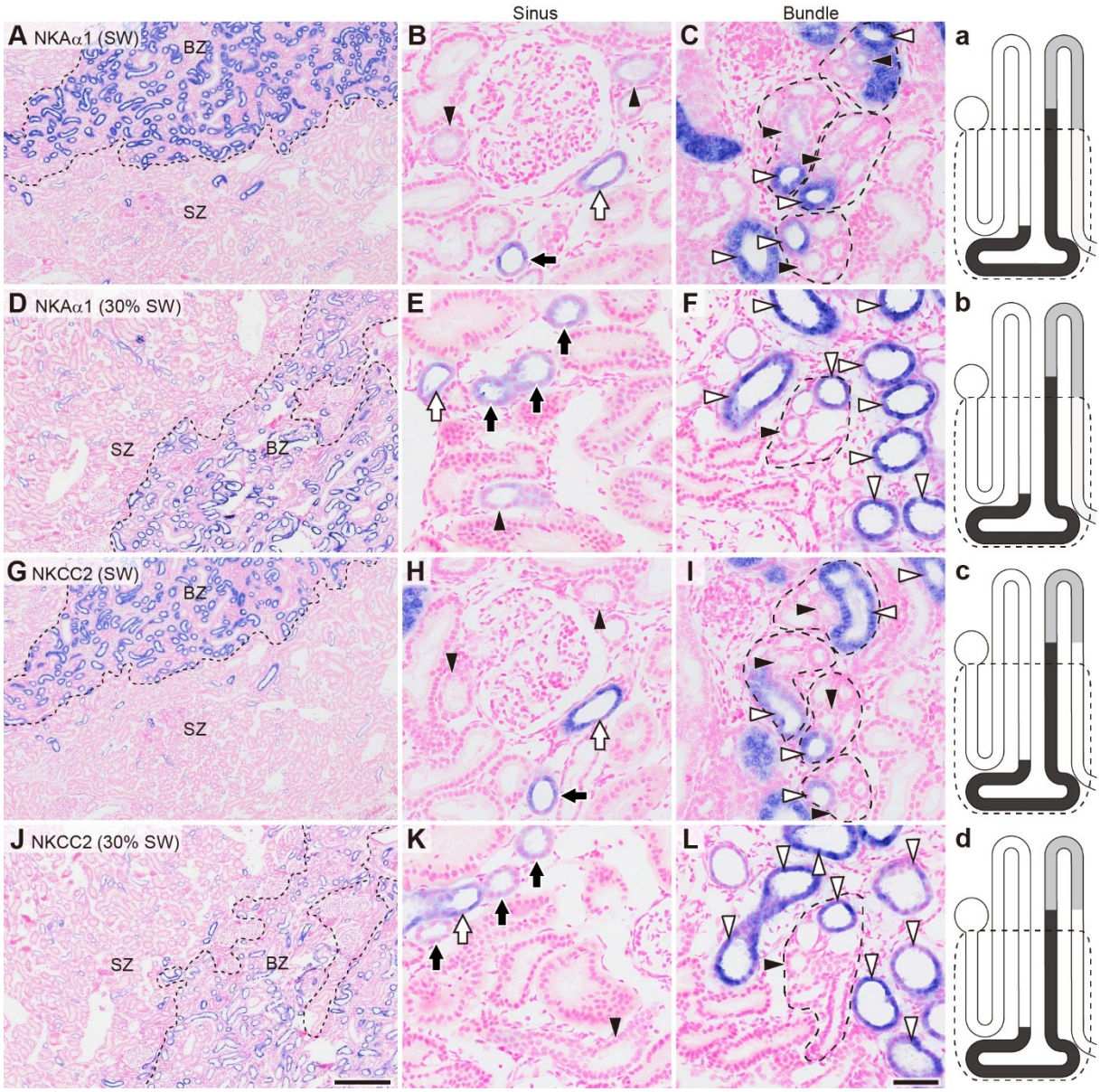

Fig. 8. *In situ* hybridization analyses of mRNAs encoding NKAα1, NKCC2, NCC and UT in the kidneys of houndsharks acclimated to SW and 30% SW. NKAα1 (A-F), NKCC2 (G-L), NCC (M-R) and UT (S-X) mRNAs in SW- (A-C, G-I, M-O, S-U) and 30% SW- (D-F, J-L, P-R, V-X) acclimated houndsharks. B, E, H, K, N, Q, T, W and C, F, I, L, O, R, U, X are magnified views of sinus zone (SZ) and bundle zone (BZ), respectively. The localization of each mRNA in the nephron is schematically illustrated (a-h). Open arrows, filled arrows, and open arrowheads represent the transitional segment from the EDT to the LDT, the LDT, and the EDT,

respectively. Filled arrowheads represent the transitional segment from the LDT to the CT in the sinus zone, and the CT in the bundle zone. Sections were counterstained with Nuclear Fast Red. Bar = 400 μm (A, D, G, J, M, P, S and V) and 50 μm (B, C, E, F, H, I, K, L, N, O, Q, R, T, U, W and X).

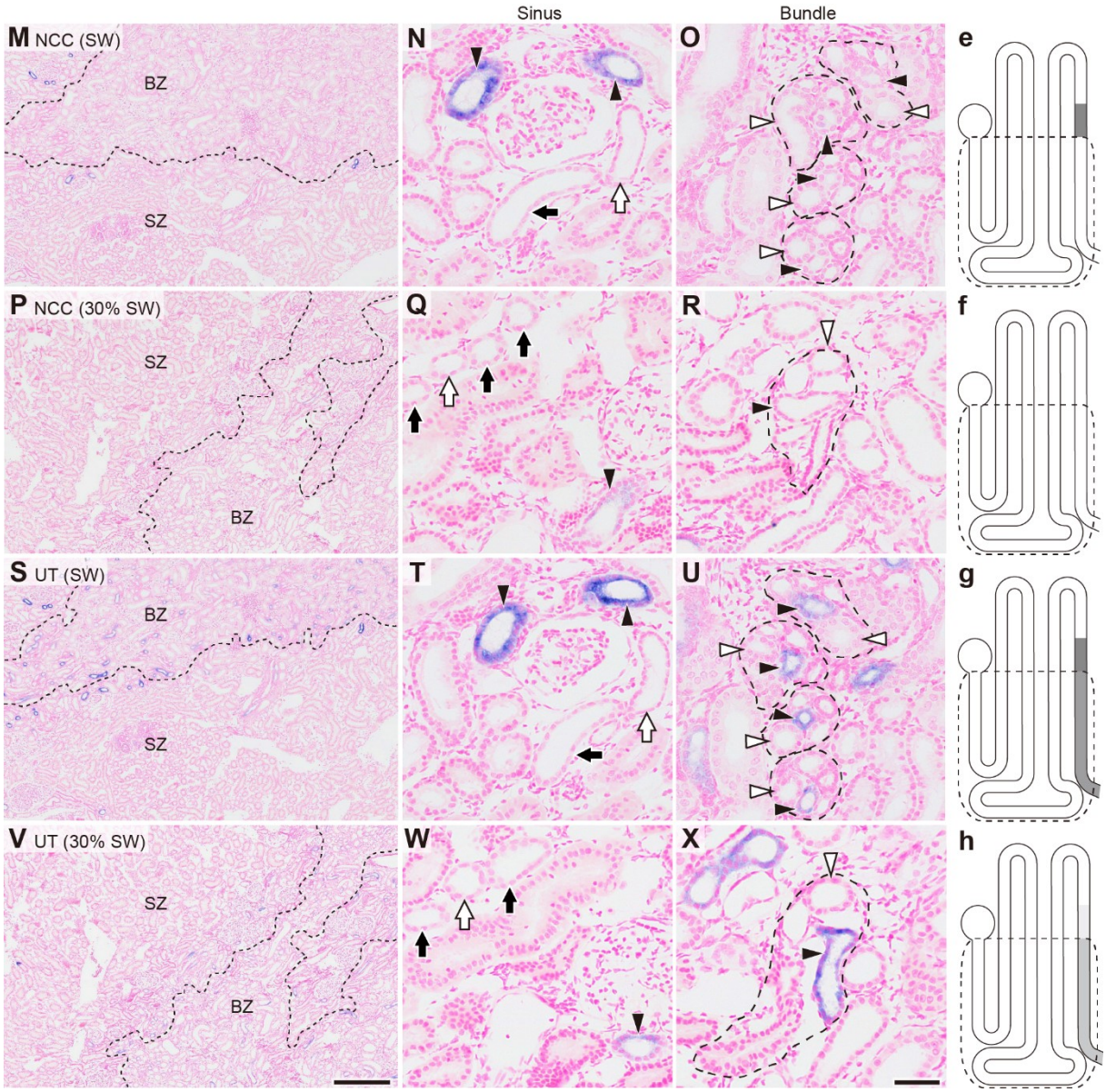

Fig. 8. Continued.

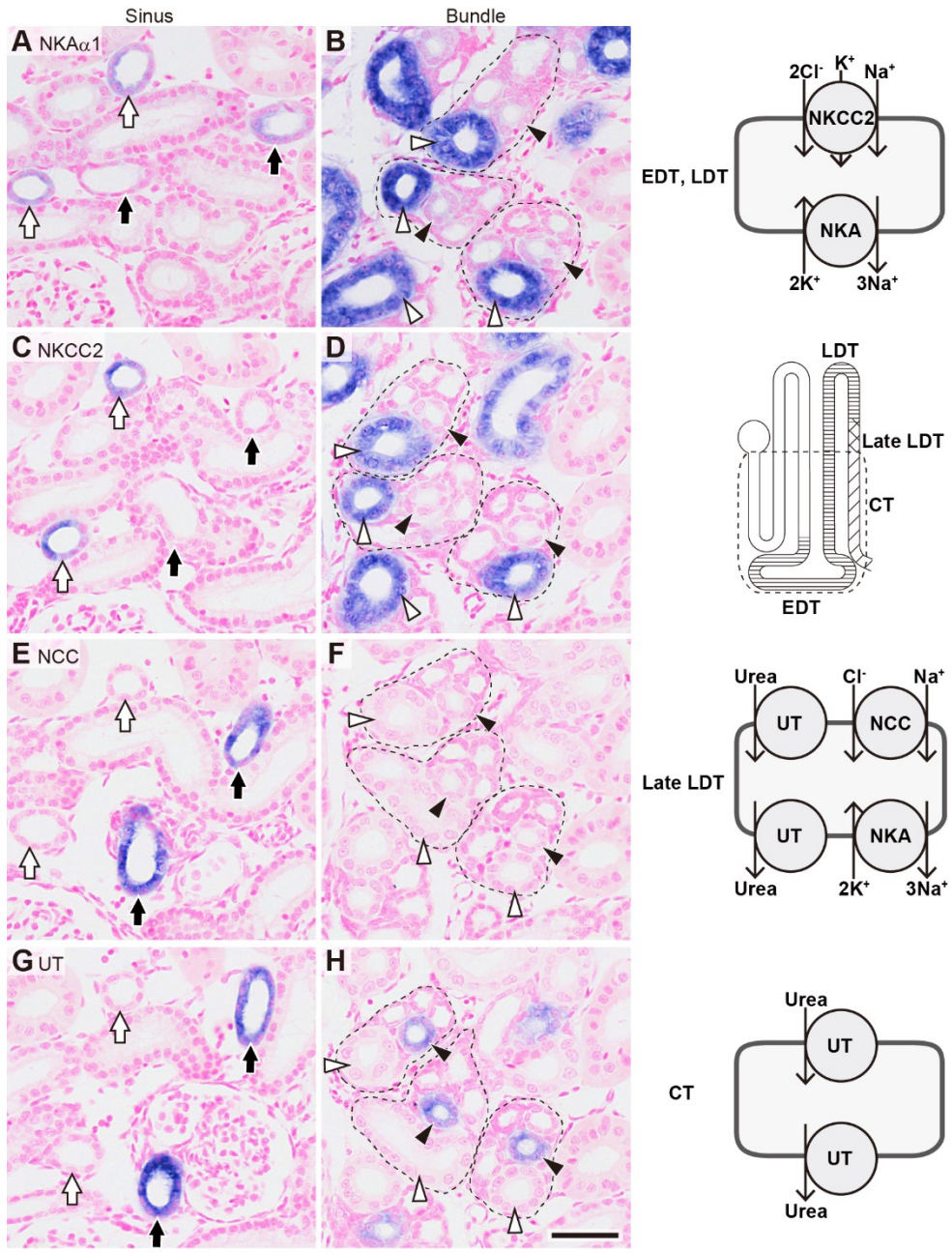

Fig. 9. Co-localization of mRNA signals in the kidney of houndshark in SW. NKAα1 (A, B), NKCC2 (C, D), NCC (E, F) and UT (G, H) mRNAs in the sinus zone (A, C, E, G) and bundle zone (B, D, F, H). Open arrows and filled arrows in the sinus zone represent the LDT and the transitional segment from the LDT to the CT, respectively. Filled arrowheads and open arrowheads represent the CT and the EDT, respectively. Sections were counterstained with Nuclear Fast Red. Bar = 50 μm.

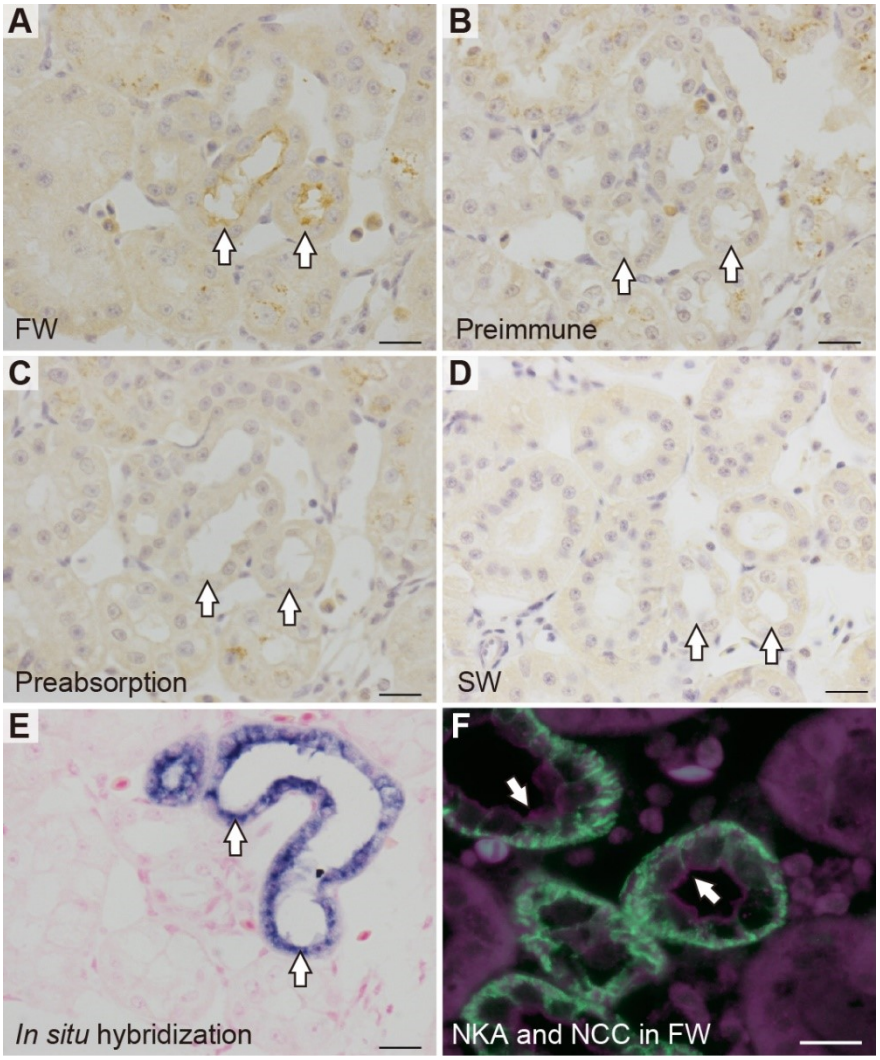

Fig. 10. Immunohistochemistry of NCC in the kidney of FW bull shark. Signals were localized on the apical membrane of the LDT (A, open arrows). The use of preimmune serum (B) and pre-absorbed antiserum (C) resulted in the disappearance of the immunoreactive signals. Signals were not observed in the kidney of SW individual (D). E, *In situ* hybridization of NCC mRNA in the kidney of FW bull shark showing the co-localization of immunoreactive signals (A) and mRNA signals (E) in adjacent sections. Sections were counterstained with hematoxylin (A-D) or Nuclear Fast Red (E). F, Double-labelling fluorescence immunohistochemistry with anti-NCC antibody (magenta; indicated by arrows) and anti-NKA antibody (green). Bars = 20 μm.

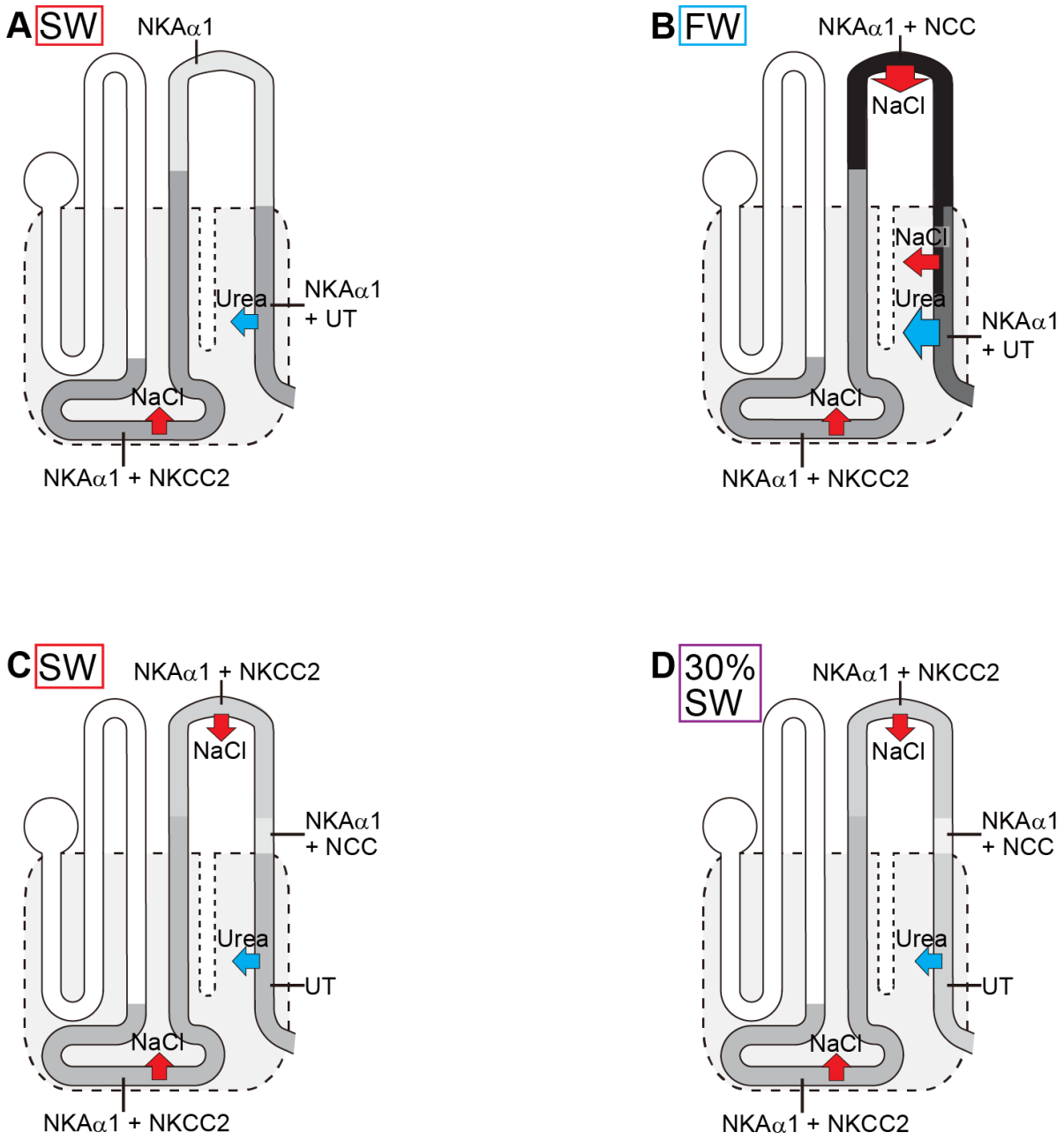

Fig. 11. Schematic diagram showing changes in kidney function among SW- (A) and FW-acclimated (B) bull sharks and SW- (C) and 30% SW-acclimated (D) houndsharks. Note that reabsorption of NaCl is enhanced in FW-acclimated bull shark kidney by the up-regulated expression of NCC and NKAα1 in the LDT and the anterior part of the CT (shown in black). The enhanced reabsorption of NaCl in the CT may contribute to urea retention. In contrast, reabsorption of NaCl is not enhanced in 30% SW-acclimated houndshark kidney.

第１章　第２節（pp. 64 - 86）

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第１章　第３節（pp. 87 - 112）

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第２章（pp. 113 - 142）

本章の内容は、学術雑誌論文として出版する計画があるため公表できない。５年以内に出版予定。

総合考察（pp. 143 - 156）

総合考察の内容は、学術雑誌論文として出版する計画があるため公表できない。５年以内に出版予定。

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論文の内容の要旨

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論文題目

Studies on the kidney function in environmental adaptation of euryhaline bull shark, *Carcharhinus leucas*
（広塩性軟骨魚オオメジロザメの環境適応における腎機能の研究）

軟骨魚類は、現生の脊椎動物顎口類のなかで最も早く分岐した分類群であり、約 1200 種が確認されている。サメやエイを含む板鰓類とギンザメを含む全頭類に分けられ、板鰓類の 95%および全ての全頭類が海生種である。海洋生態系の頂点捕食者として重要な動物群である一方で、乱獲や環境変化による個体数の減少は著しく、30%以上の板鰓類が絶滅危惧種あるいは準絶滅危惧種に指定されていることからも、軟骨魚類の生理・生態の理解は喫緊の課題である。軟骨魚類は、海という高浸透圧環境に適応するためのメカニズムもユニークであり、体内に高濃度の尿素を蓄積することで体液浸透圧を海水よりわずかに高く保ち、脱水から免れている。このことは、飲水と鰓からの塩の排出により海水の約 1/3 の体液浸透圧を保つ海生真骨魚類とは大きく異なる。尿素による体液調節を行うため、軟骨魚類の体液調節器官は独自の進化を遂げてきた。たとえば海生種の腎臓は 4 回のループを含む複雑なネフロン構造を持つことが知られ、これにより尿の生成過程で 90%以上もの尿素を再吸収する。

前述の通り、ほとんどの軟骨魚類は海生種であるが、板鰓類の約 5%の種は淡水域にも生息することが知られている。オオメジロザメ (*Carcharhinus leucas*) はサメ類の中でほぼ唯一の広塩性種であり、その生活史の中で海水域と淡水域を行き来すると考えられている。オオメジロザメは海水中では他の海生種と同様に尿素を利用した体液調節を行う一方で、淡水に進入しても高濃度のイオンと尿素を保持するため、その体液浸透圧は 600 mOsm 以上であり、これは真骨魚の約 2 倍という高い値である。淡水環境のみに生息するポタモトリゴン属の淡水エイが尿素を持たず、真骨魚と同様の体液組成を持つことからも、オオメジロザメをはじめとする広塩性板鰓類の体液組

成が、いかに特殊であるかがわかる。淡水環境下では体内外の大きな浸透圧差により、水の流入と塩や尿素の喪失が問題となるが、どのような仕組みにより淡水環境に適応できるのか、そのメカニズムはほとんどわかっていない。広塩性のメカニズムの解明は、軟骨魚類の体液調節機構の理解とともに、板鰓類の生息環境の拡大という観点からも重要である。

淡水環境では浸透圧差により体内に水が流入するが、これを排出できる唯一の器官が腎臓である。軟骨魚の腎臓は、海水中では過剰なイオンの排出と尿素の保持に関わるが、淡水中ではイオンや尿素を保持しながら大量の水を排出しなければならない。すなわち、淡水環境への移行により機能が大きく変化すると考えた。そこで腎臓に注目し、第一章ではオオメジロザメを淡水移行させたときに発現が変化する遺伝子を網羅的に探索し、特に 3 つの分子に注目して広塩性のメカニズムの解析を行った。第二章では、西表島の浦内川で捕獲調査と河川環境調査を行い、第一章で見出したメカニズムが実際の河川環境でもはたらいているかどうかを確かめた。

第一章第一節　オオメジロザメの広塩性に寄与する遺伝子の網羅的探索と機能解析１；Na^+-Cl^- 共輸送体は本種の淡水適応に重要な分子である

美ら海水族館で実施した淡水移行実験の結果、オオメジロザメが淡水中でも高濃度の NaCl と尿素を保持し続けることが確認された。アトランティックスティングレイ (*Hypanus sabina*) における先行研究で測定された糸球体濾過量と尿量を参考に、今回のオオメジロザメのネフロンにおける再吸収量を計算した結果、淡水移行により NaCl の再吸収量が少なくとも 1.5 倍に増加したことが示唆された。理化学研究所と共同で行った RNA-sequencing により、淡水への移行により発現が増加した遺伝子を 138 種類見出した。それらの中から、物質輸送に重要な膜輸送体である溶質輸送体 (slc) ファミリーに注目したところ、Na^+-Cl^- 共輸送体 (slc12a3, NCC) の発現が淡水個体の腎臓で大きく上昇することを見出した。NCC の発現は、淡水個体のネフロンの遠位尿細管後部 (LDT) と集合細管 (CT) に検出され、海水個体ではその発現は非常に低かった。NCC は LDT を構成する細胞の頂端膜に局在し、NaCl 輸送の駆動力を生み出す Na^+/K^+-ATPase (NKA)の発現が同一細胞に認められたことから、淡水環境への移行により NCC が特に LDT において NaCl の再吸収を行うようになることがわかった。一方で、移行に伴う NCC と NKA の発現上昇は、尿素再吸収の場と考えられている CT においても観察された。現在提唱されているモデルでは、尿素再吸収の第一段階として NaCl の再吸収が必要と考えられており、CT における NCC と NKA の発現上昇は、NaCl の保持だけでなく、尿素の保持にとっても重要だと考えている。このような NCC をはじめとする発現量ならびに発現領域の変化は、狭塩性種であるドチザメ (*Triakis scyllium*) を希釈海水に移行させた時には見出されず、NCC は LDT 終端部という限られた領域に弱い発現が認

められるだけであった。以上の結果から、オオメジロザメ腎ネフロンの LDT と CT は、淡水環境への移行により NaCl ならびに尿素の再吸収を亢進し、オオメジロザメの広塩性に重要な役割を果たすことが示唆された。そして、このメカニズムにおいて NCC が鍵となる分子であることが明らかとなった。

第一章第二節　オオメジロザメの広塩性に寄与する遺伝子の網羅的探索と機能解析２；淡水移行個体の腎臓における上皮性 Na^+チャネル mRNA の発現

哺乳類の腎ネフロンにおいて、NCC は遠位部において上皮性 Na^+チャネル (ENaC) とともに発現することが知られている。RNA-sequencing の結果からは、発現が上昇した遺伝子の中に ENaC は存在しなかったが、定量 PCR による解析では淡水移行により発現が有意に上昇することがわかった。ENaC はα, β, γという 3 つのサブユニットから構成されており、Na^+輸送活性を有する ENaCα は海水個体では CT の前半部にのみ発現し、LDT での発現は極めて弱かった。淡水移行により ENaCαは LDT 全体に強く発現し、CT の後半にも発現するようになった。輸送活性の調節に関わる ENaCβと ENaCγの発現も LDT で検出され、淡水移行による発現量の変化は見られなかったものの、CT の前半部でも発現するようになった。以上の結果から、ENaC もオオメジロザメの淡水適応に重要な分子であることが示唆された。

第一章第三節　オオメジロザメの広塩性に寄与する遺伝子の網羅的探索と機能解析３；海水環境におけるオオメジロザメの腎臓遠位尿細管後部における排出機能

ここまでの結果から、腎ネフロンの LDT と CT がオオメジロザメの淡水環境での生存に重要な分節であることが示唆された。一方で、海水環境における LDT の役割は不明であった。RNA-sequencing では淡水移行により発現が低下する遺伝子が 75 種類見出されており、Slc ファミリー遺伝子も 3 種類存在した。このうち Slc4a11 mRNA が海水個体の LDT 全体に強く発現し、淡水個体では NCC と入れ替わるように発現が消失した。アフリカツメガエル卵母細胞に slc4a11 遺伝子を発現させて機能解析を行った結果、ホウ酸輸送活性を持つことが示された。NaCl 再吸収分節としてオオメジロザメの淡水適応に重要な役割を果たす LDT は、海水中ではホウ酸などの不要物質の排出を担い、環境変化に伴う LDT の機能切替が本種の広塩性能力に重要であることが示唆された。

第二章　西表島浦内川に生息するオオメジロザメのフィールド調査

第一章において、オオメジロザメの広塩性能力には腎臓の LDT と CT という領域における NCC や ENaC、Slc4a11 の発現制御が重要であることがわかった。このような現象が実際の河川に生息する個体でも起こっているのかを明らかにするために、沖縄県西表島の浦内川に遡上する野生個体の捕獲調査と遺伝子発現量の解析を行った。2014 年 5 月から 2015 年 9 月、2016 年 6 月から 8 月、2017 年と 2018 年の 6 月の各月 1 回、河口から 4 km, 7 km, 8 km の 3 ヶ所で刺し網調査を行った。生後間もない個体の新規加入は 6 月からみられ、水温の低下する 10 月以降捕獲されなかった。浦内川では、河口から 8 km 上流まで川底に海水が入り込む塩水楔が発生し、川底まで完全な淡水だった時には捕獲されなかった。使用した刺し網は、塩水楔が存在する川底から 2 メートル立ち上がる仕掛であるため、川底にサメがいたと仮定したところ、浦内川のオオメジロザメは汽水環境ではあるものの、血漿よりも低浸透圧な環境に生息することが確認された。第一章で解析した遺伝子を含む 11 種類の遺伝子発現量を調べた結果、NCC の発現量が海水群と淡水群の中間の値となる等、汽水環境に生息することが遺伝子発現からも示唆された。

本研究により、オオメジロザメの広塩性を可能にするメカニズムとして、腎ネフロンの LDT と CT の機能切替・亢進が示唆され、その鍵となる分子として NCC や ENaC、Slc4a11 などが示された。浦内川においては、低浸透圧環境に生息するものの、完全な淡水環境を好むわけではないことも示唆され、このことはオオメジロザメの体液組成を考えれば納得がいくものである。オオメジロザメは広塩性という特異な能力を持つことから多くの研究者の注目を集めてきたが、飼育実験の困難さから広塩性のメカニズムは不明であった。本研究では、飼育実験個体を用いるラボワークと、実際の河川でのフィールドワークの両面から研究を進めたことで、広塩性のメカニズムの解明に進展をもたらしただけでなく、本種の生息環境の理解にもつながり、軟骨魚類の環境適応の理解に対して大きく貢献するものである。

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