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Osmoregulation in elephant fish *Callorhinchus milii* (Holocephali), with special reference to the rectal gland

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Summary

Osmoregulatory mechanisms in holocephalan fishes are poorly understood except that these fish are known to conduct urea-based osmoregulation as in elasmobranchs. We, therefore, examined changes in plasma parameters of elephant fish *Callorhinchus milii*, after gradual transfer to concentrated (120%) or diluted (80%) seawater (SW). In control fish, plasma Na and urea concentrations were about 300 mmol l^{-1} and 450 mmol l^{-1}, respectively. These values were equivalent to those of sharks and rays, but the plasma urea concentration of elephant fish was considerably higher than that reported for chimaeras, another holocephalan. After transfer to 120% SW, plasma osmolality, urea and ion concentrations were increased, whereas transfer to 80% SW resulted in a fall in these parameters. The rises in ion concentrations were notable after transfer to 120% SW, whereas urea concentration decreased predominantly following transfer to 80% SW. In elephant fish, we could not find a discrete rectal gland. Instead, approximately 10 tubular structures were located in the wall of post-valvular intestine. Each tubular structure was composed of a putative salt-secreting component consisting of a single-layered columnar epithelium, which was stained with an anti-Na^+,K^+-ATPase serum. Furthermore, Na^+,K^+-ATPase activity in the tubular structures was significantly increased after acute transfer of fish to concentrated SW (115%). These results suggest that the tubular structures are a rectal gland equivalent, functioning as a salt-secreting organ. Since the rectal gland of elephant fish is well developed compared to that of Southern chimaera, the salt-secreting ability may be higher in elephant fish than chimaeras, which may account for the lower plasma NaCl concentration in elephant fish compared to other chimaeras. Since elephant fish have also attracted attention from a viewpoint of genome science, the availability of fish for physiological studies will make this species an excellent model in holocephalan fish group.

Key words: holocephalan fish, osmoregulation, rectal gland, elephant fish.

Introduction

To overcome hyperosmotic stress in the marine environment, cartilaginous fish maintain their plasma iso-osmotic or slightly hyper-osmotic to surrounding seawater, primarily through the retention of urea (ureosmotic). This osmoregulatory system was initially considered unique to cartilaginous fish, but an increasing body of evidence suggests that the ureosmotic strategy is widely distributed throughout vertebrate species. For instance, the coelacanth is a well-known bony fish conducting urea-based osmoregulation (Mommsen and Walsh, 1989), and the crab-eating frog is a brackish water inhabitant that uses urea to adjust its plasma to the brackish environment (Wright et al., 2004). In the mammalian kidney, a high concentration of urea is maintained in the inner medulla to create a high osmolality environment in the interstitium; water is reabsorbed from primary urine into the interstitium by an osmotic gradient, resulting in the retention of water in the body (Sands, 2004). These facts suggest that the ureosmotic strategy is a basic mechanism in vertebrates for retention of water in high salinity and arid environments. Therefore, understanding of the evolution of the ureosmotic system is an important aspect of vertebrate osmoregulation.

Elephant fish and chimaeras belong to the Holocephali, a primitive group in the Chondrichthyes (Compagno, 2005; Patterson, 1965). Little is known about osmoregulatory mechanisms in holocephalan fishes except that they conduct urea-based osmoregulation as in elasmobranchs. Interestingly, in previous reports, plasma ion levels of chimaeras were found

to be higher, and urea levels lower, than those of elasmobranchs (Fange and Fugelli, 1962; Fange and Fugelli, 1963; Read, 1971; Rasmussen, 1974; Urist and Van de Putte, 1967), implying that the osmoregulatory systems of holocephalan fishes represent primitive traits of the urea-based osmoregulation in cartilaginous fish. In general, however, holocephalan fishes are deep-sea inhabitants (Didier, 2004), so that physiological investigation has been considered to be difficult to perform in this group. The elephant fish *Callorhinchus milii* is the Australian and New Zealand representative of the Callorhinchidae family (Last and Stevens, 1994). Elephant fish inhabit continental shelf waters to depths of at least 200 m; however, they display a clear seasonal migration into bays in south-eastern Australia and Tasmania during the egg-laying season. Thus, they are one of the only holocephalan species on which we can perform physiological studies, and therefore, they represent an excellent model to study the evolution of the ureosmotic system in cartilaginous fishes.

In the present study, we determined plasma parameters in healthy elephant fish, and then examined the effects of transfer to different environmental salinities. Unlike elasmobranchs, elephant fish do not have a discrete rectal gland. Instead, we found rectal gland-like structures in the wall of the post-valvular intestine. We then examined morphological and functional characteristics of the gland and determined that those structures actually function as the salt-secreting rectal gland.

Materials and methods

Fish

Elephant fish *Callorhinchus milii* Bory de St Vincent of both sexes (body mass 0.93–3.15 kg) were collected from Westernport Bay, Victoria, Australia, using recreational fishing equipment consisting of breaking strain line and a hook baited with pilchard *Sardinops neopilchardus*. Fish were also collected from commercial fishermen in Port Phillip Bay using a haul seine with stretched mesh wings and a stretched mesh bag. Fish were transported to Primary Industries Research Victoria, Queenscliff Centre, using a fish transporter (1000 l). In the first experiment, they were kept in a 20 kl round tank with running seawater (SW) under a natural photoperiod for at least 2 weeks before the experimentation, and fed daily with pilchard. In the second experiment, they were kept unfed in an 8 kl round tank for at least 3 days before the experiment. All animal experiments were conducted according to the Guideline for Care and Use of Animals approved by the committees of University of Tokyo and Deakin University.

Transfer experiment

Experiment 1

In March 2004, the transfer experiment to concentrated (120%) or diluted (80%) SW was performed. For this experiment, 5 kl round tanks with 3 kl of aerated SW were used. Three fish were transferred from the holding tank to each experimental tank. On the next day (day 0), full-strength (100%) SW, freshwater, and 200% SW was added to each tank to produce 100% SW, 95% SW and 105% SW, respectively. On days 1, 2 and 3, the same degree (5%) of salinity change was performed; salinity was gradually (5% each day) increased and decreased to 120% and 80%, respectively. The same experiment was repeated to increase the number of fish for analysis. The 200% SW was prepared by dissolving artificial SW salts in SW.

On day 4 (1 day after reaching to 120% or 80%), fish were sampled between 10:00 h and 14:00 h. After anaesthetization in 0.02% (w/v) 3-aminobenzoic acid ethyl ester (Sigma, St Louis, MO, USA), blood samples were obtained from the caudal vasculature by syringe and centrifuged at 2250 ***g*** for 10 min to obtain plasma; plasma was stored at –20°C until analysis. Plasma osmolality was measured with a vapor pressure osmometer (Wescor 5520, Logan, UT, USA), and plasma sodium and chloride concentrations were measured using an atomic absorption spectrophotometer (Hitachi Z5300, Tokyo, Japan) and a chloride meter (Buchler Instruments Inc., Lenexa, KS, USA), respectively. Plasma urea levels were measured by the enzymatic colorimetric method using the Wako Urea NB test (Wako Pure Chemical Industries, Japan). Post-valvular intestines were dissected out, and were fixed in 4% paraformaldehyde in 0.05 mol l^{-1} phosphate buffer (pH 7.4). The osmolality of the fixative was adjusted at approximately 1000 mOsm with NaCl and urea.

Experiment 2

From March to April 2005, time-course sampling after acute transfer to concentrated SW (approximately 115%) was performed. For this experiment, two 5 kl round tanks with 3 kl of aerated SW were used. Five fish were transferred from the holding tank to each experimental tank. On the next day, three fish were sampled as the day 0 group. After sampling, 200% SW was added to produce 115% SW. On days 1, 2 and 3 after changing to 115% SW, two or three fish were sampled. The same experiment was repeated to increase the number of fish. Plasma parameters were obtained by the same methods as the first experiment. For the measurement of Na^+,K^+-ATPase activity, the tubular structures embedded in the post-valvular intestine were dissected out, cut into small pieces, and stored in 200 μl of buffer containing 150 mmol l^{-1} sucrose, 10 mmol l^{-1} Na_2-EDTA and 50 mmol l^{-1} imidazole (SEI buffer) at –80°C until analysis.

Histochemical analyses

The post-valvular intestine was fixed at 4°C for 2 days, washed twice in 70% ethanol, and then embedded in Paraplast (Kendall, Mansfield, MA, USA). Transverse and sagittal sections were cut at 7–10 μm, mounted onto gelatin-coated slides and processed for histochemical analyses. Immunohistochemical staining for Na^+,K^+-ATPase was performed with the avidin–biotin–peroxidase complex kit (Vector, Burlingame, CA, USA) (Hyodo et al., 2004a). In brief, after rehydration, tissue sections were incubated sequentially with: (1) 2% normal goat serum in phosphate-buffered saline (pH 7.4; PBS–NGS) for 2 h at room temperature, (2) the anti-

Na^+,K^+-ATPase α-subunit serum diluted 1:2000 with PBS–NGS for 48 h at 4°C, (3) biotinylated, goat anti-rabbit IgG for 30 min at room temperature, (4) avidin–biotin–peroxidase complex for 45 min at room temperature, and (5) 0.05% diaminobentizine tetrahydrochloride (Sigma) and 0.01% hydrogen peroxide in 50 mmol l^{-1} Tris buffer (pH 7.2) for 10 min at room temperature. Stained sections were counterstained with Haematoxylin. The anti-Na^+,K^+-ATPase serum (a gift from Prof. K. Yamauchi, Hokkaido University, Japan) was raised against a synthetic peptide which represents a completely conserved sequence among all vertebrate groups examined including elasmobranchs (Ura et al., 1996; Hyodo et al., 2004a). Adjacent sections were stained for neutral mucins using periodic acid–Schiff (PAS).

Light micrographs were obtained using a digital camera (DXM1200; Nikon, Tokyo, Japan). Images were morphometrically analyzed with NIH ImageJ (http://rsb.info.nih.gov/ij/) to quantify the thickness and area of rectal gland-like structures.

Measurement of Na^+,K^+-ATPase activity

Rectal gland Na^+,K^+-ATPase activity was measured by a micro-assay method (Katoh et al., 2003; McCormick, 1993). After 100 μl of SEI buffer containing 0.3% sodium deoxycholic acid was added, a piece of rectal gland-like tissue stored in 200 μl of SEI buffer was homogenized with a Kontes pellet pestle on ice, and centrifuged at 3000 ***g*** for 30 s to remove insoluble materials. The supernatant was assayed for Na^+,K^+-ATPase activity and protein content. Homogenate samples (10 μl) were placed in the wells of a 96-well plate in quadruplicate. The assay mixture [50 mmol l^{-1} imidazole, 2.8 mmol l^{-1} phosphoenolpyruvate, 0.22 mmol l^{-1} NADH, 0.7 mmol l^{-1} ATP, 4 i.u. ml^{-1} lactic dehydrogenase and 5 i.u. ml^{-1} pyruvate kinase, 47 mmol l^{-1} NaCl, 5.25 mmol l^{-1} $MgCl_2$ and 10.5 mmol l^{-1} KCl (200 μl)] with or without 0.5 mmol l^{-1} ouabain was added to the wells in duplicate just before reading the absorbance at a wavelength of 340 nm. The linear rate of NADH disappearance was measured every 20 s up to 10 min at 25°C. The protein content of the sample was determined using a BCA Protein Assay Kit (Pierce, IL, USA). The Na^+,K^+-ATPase activity was calculated as the difference in ATP hydrolysis between the presence and absence of ouabain, and expressed as μmol ADP mg $protein^{-1}$ h^{-1}.

Statistics

All numerical data are presented as the mean ± standard error of the mean (s.e.m.). Statistical analyses were performed using ANOVA, followed by the appropriate *post-hoc* test with a software, KyPlot 5.0 (KyensLab Inc., Tokyo, Japan).

Results

Plasma parameters of elephant fish after transfer to different salinities

In the first experiment, we examined the effects of transfer to different environmental salinities on body fluid homeostasis. Table 1 shows plasma parameters in elephant fish after gradual transfer to concentrated (120%) and diluted (80%) SW for 4 days (5% change per day). In control fish, plasma was nearly iso-osmotic to full-strength SW (1069 mOsm kg^{-1}). Plasma Na^+ and Cl^- concentrations of control fish were lower than those reported in a holocephalan fish, *Chimaera monstrosa* (Fange and Fugelli, 1962; Fange and Fugelli, 1963), or similar to those of another chimaera species, *Hydrolagus colliei* (Read, 1971; Urist and Van de Putte, 1967). By contrast, plasma urea values of control elephant fish were considerably higher than those reported in chimaera species (Table 1). Urea accounts for nearly half of the plasma osmolality in elephant fish. Following transfer of elephant fish from full-strength SW to 120% SW (1308 mOsm kg^{-1}), there were significant increases in osmolality, urea and ion concentrations (Table 1). Transfer to diluted SW (80%, 824 mOsm kg^{-1}) resulted in a fall in these parameters, but the degree of changes in the osmolytes was different compared with 120% SW. The increases in Na and Cl ion concentrations were larger than that of urea after transfer

Table 1. *Changes in plasma parameters after gradual transfer to concentrated (120%) and diluted (80%) seawater (SW)*

%SW	*N*	Osmolality (mOsm kg^{-1})	Ion concentrations (mmol l^{-1})			Na^+/Osm (%)	Urea/Osm (%)
			Na^+	Cl^-	Urea		
100	6	1057.3±3.6	309.4±4.5	285.5±2.0	472.5±16.3	29.3±0.5 (28.2±0.4)*	44.7±1.5 (37.5±0.6)*
120	6	1260.7±14.4	383.9±9.4	357.7±6.0	522.8±15.6	30.5±0.8 (28.9±0.4)*	41.4±0.8 (36.6±0.5)*
80	4	886.0±0.7	284.3±5.7	263.5±5.9	345.6±11.8	32.1±0.6 (28.8±0.3)*	39.0±1.3 (35.8±0.5)*
Chimaera monstrosa[1]			362.5	380	265.5		
Hydrolagus colliei[2]			268.0	272.0	303.0		
Hydrolagus colliei[3]			300±32	306±28	245±27		
Callorhynchus milii[4]				230	400		

*Dogfish shark *Triakis scyllium* data (Hyodo et al., 2004b).
[1](Fange and Fugelli, 1963); [2](Urist et al., 1967); [3](Read, 1971); [4](Dakin, 1931).

to 120% SW. Meanwhile, urea concentration decreased significantly following transfer to diluted SW, which resulted in an increased Na^+/osmolality ratio and a decreased urea/osmolality ratio (Table 1). In a dogfish, *Triakis scyllium*, these ratios were maintained even after transfer to 130% SW or 70% SW (Table 1) (Hyodo et al., 2004b).

Comparative morphological study of rectal gland-like tissues in elephant fish and Southern chimaera

In the elephant fish body cavity, we could not find a discrete salt-secreting gland that could be ascribed as a rectal gland. Instead, approximately a dozen tubular structures were located in the wall of post-valvular intestine (Fig. 1A,B). The tubular structures began at the termination of the well-developed spiral intestine (arrow in Fig. 1A) and extended posteriorly to the middle part of the post-valvular intestine (rectum) (Fig. 1A,B). In one fish (63 cm in fork length), the tubular structures were 8–10 mm in length, and were around the whole rectal wall. The width and thickness of the structure in the wall was over 2.5 mm. Each tubular structure was subdivided into multiple lobes, giving a nodular appearance (Fig. 1C).

For the purpose of comparison, the identical organ was also examined in another holocephalan fish, Southern chimaera [*Chimaera* sp. A (Last and Stevens, 1994) Fig. 1D,E]. The glandular organ of Southern chimaera had similar morphological characteristics as that of elephant fish; however, the tubular structures in the chimaera appeared to be less developed compared with those in elephant fish (Fig. 1B,D). The width and thickness of each tubular structure was 0.7–1.3 mm, and they were considerably thinner than the homologous structures in elephant fish. The lobes are round and formed clusters that gave the appearance of a multi-lobulated gland (Fig. 1E).

Figs 2 and 3 show longitudinal and transverse sections of the tubular structures. In both elephant fish and Southern chimaera, each lobe can be clearly divided into two layers. The outer layer is composed of small epithelial tubules that are similar to the salt-secreting tubules in the rectal gland of elasmobranchs (Fig. 2A and Fig. 3A). However, the tubules in holocephalan fishes are randomly oriented compared with those in elasmobranchs. The tubules consist of a single-layered columnar epithelium that is intensely stained by anti-Na^+,K^+-ATPase serum (Fig. 2A, Fig. 3A,C). The tubules are tightly packed, and interspersed with capillaries or sinusoidal vasculatures, so that red blood cells are visible in the extratubular space. Na^+,K^+-ATPase immunoreactive signals were localized on the basolateral membranes of epithelial cells (Fig. 3D).

In contrast to the outer layer, the inner layer of each lobe is composed of tubules lined with large, cuboidal mucous cells, which stained intensely with PAS but not with anti-Na^+,K^+-ATPase serum (Fig. 2A,B and Fig. 3A,B). In the outer layer, mucous cells were not found, whereas the inner layer was exclusively composed of mucous cells (Figs 2 and 3). In the boundary zone between the outer and inner layers, a transition from Na^+,K^+-ATPase-positive epithelia (presumably secretory epithelia) to mucous cell-type epithelia was observed along a single tubule (arrows in Fig. 3C,D). The mucous cells form large, cylindrical lumina that connect with one another, forming a collecting duct. Individual collecting ducts from adjacent lobular glands merge together; a central duct (asterisks in Figs 2 and 4) passes posteriorly and finally opens to the intestinal lumen (arrows in Fig. 4). Sections of the lobular gland of Southern chimaera showed the same characteristics as those of elephant fish (Fig. 2C). The gland appeared to be thinner in chimaera compared with elephant fish, indicating that, again, the gland is less-developed in chimaera species.

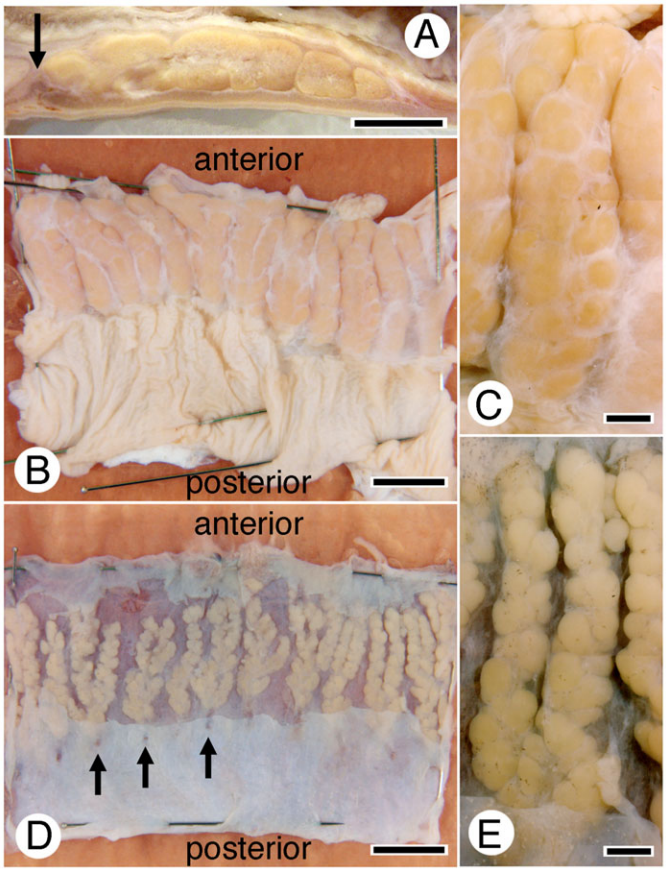

Fig. 1. Gross anatomy of the rectal part of the intestine in elephant fish (A–C) and Southern chimaera (D,E). (A) A longitudinal slice of the post-valvular intestine. The arrow indicates the anterior end of glandular tissue at the termination of valvular intestine. (B,D) Whole-mount view of the glandular tissue of elephant fish (B) and Southern chimaera (D). Luminal epithelia and inner muscle layers were removed to expose the glandular tissue. Arrows in D indicate openings of the glandular tissues. (C,E) Magnified view of the glandular tissue in B and D, respectively. Scale bars, 0.5 mm (A); 1 mm (B,D); 200 μm (C,E).

Plasma parameters and Na^+,K^+-ATPase activities following acute transfer to a high salinity environment

In the second experiment, fish were acutely transferred to 115% SW by adding concentrated SW, and they were sampled on days 0, 1, 2 and 3. As in the first experiment, plasma

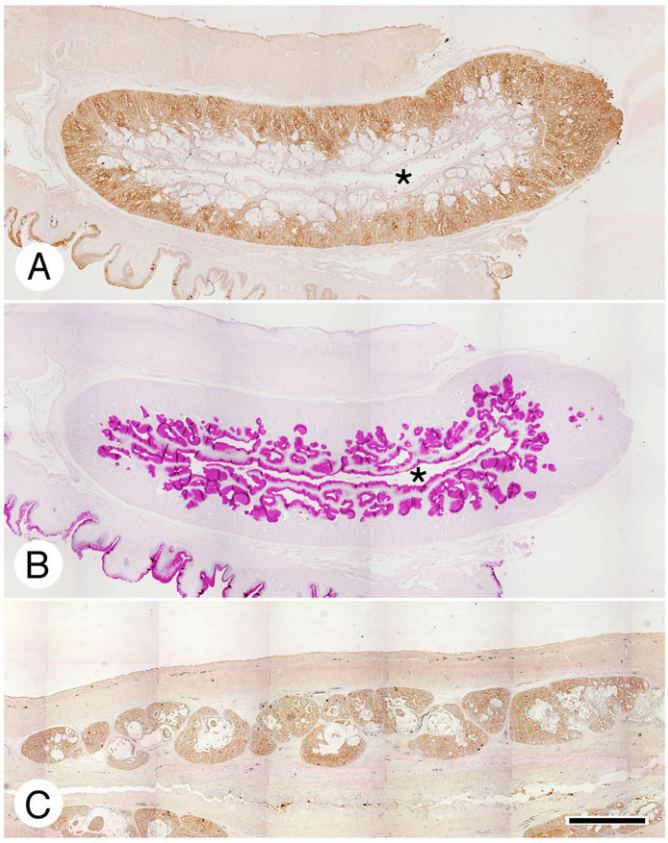

Fig. 2. Sagittal sections of the glandular structures in elephant fish (A,B) and Southern chimaera (C). Sections were stained with anti-Na^+,K^+-ATPase serum (A,C) and with periodic acid–Schiff (PAS; B). Asterisks in A and B indicate a central duct that runs along the anteroposterior axis. Scale bar, 1 mm.

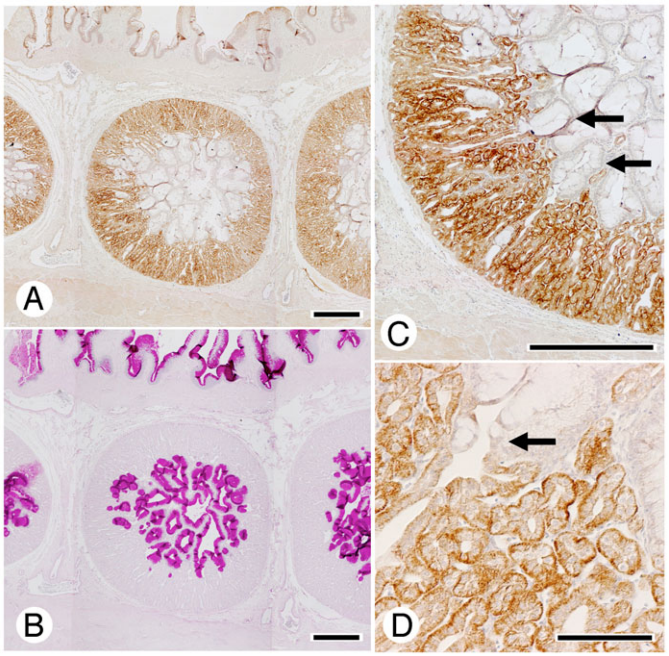

Fig. 3. Cross sections of the glandular structures in the rectal part of the intestine in elephant fish. Adjacent sections were stained with anti-Na^+,K^+-ATPase serum (A,C,D) and with periodic acid–Schiff (PAS; B). (C,D) Magnified views of the section in A, showing the transition from the secretory epithelium (stained with anti-Na^+,K^+-ATPase serum) to the mucus cells along the single tubule (arrows). Immmunoreactive signals for Na^+,K^+-ATPase were localized on the basolateral membranes but not on the apical membranes of epithelial cells. Scale bars, 500 μm (A–C); 100 μm (D).

parameters such as osmolality, ion and urea concentrations were increased by transfer to the high salinity environment (Table 2). Sodium and chloride ions increased rapidly on day 1 and the high level was maintained during the experiment, whereas plasma urea concentration increased gradually. Na^+,K^+-ATPase activity in the tubular structures was increased following the transfer to the high salinity environment; the increase was statistically significant relative to the control on day 3.

Discussion

In general, holocephalan fishes are deep-sea inhabitants. Therefore, the occurrence of large numbers of individuals of a holocephalan species, elephant fish, in shallow waters of southern Australia, Tasmania and New Zealand during the egg-laying season enable us to perform physiological studies on primitive cartilaginous fish. Using healthy, captive elephant fish, we have examined plasma osmotic parameters in control and osmotically adjusted fish and found that elephant fish conduct ureosmotic regulation for adaptation to a hyperosmotic environment. Elephant fish do not have a discrete rectal gland, typical of elasmobranchs. Instead, we found well-developed, tubular-shaped glandular tissues in the wall of the post-valvular intestine, and concluded that the tissues function as the salt-secreting rectal gland based on morphological, histochemical and enzymatic studies. Recently, elephant fish have attracted attention as a model for genome studies of cartilaginous fish (Venkatesh et al., 2005; Venkatesh et al., 2006). The haploid cellular DNA content of elephant fish was found to be 1200 Mb; this is much smaller than the known cartilaginous fish genome (3800-7000 Mb) (Hinegardner, 1976). Because of the availability of genome information and the relative ease of capturing elephant fish for physiological study, the species will serve as a useful model for the study of holocephalan fishes, the primitive cartilaginous fish group.

Only a few studies have described the osmoregulatory mechanisms of holocephalan fishes. Krukenburg (Krukenburg, 1888) was the first investigator to observe high plasma urea concentration in a holocephalan species, *Chimaera monstrosa*. His observation was verified in the family Chimaeridae: *Hydrolagus* (Hunter, 1929; Smith, 1936; Read, 1971; Rasmussen, 1974; Urist and Van de Putte, 1967) and *Chimaera* (Fange and Fugelli, 1962; Fange and Fugelli, 1963). Interestingly, in previous reports, plasma ion levels of chimaeras were higher, whereas urea levels were lower, than those of elasmobranchs, implying that the osmoregulatory

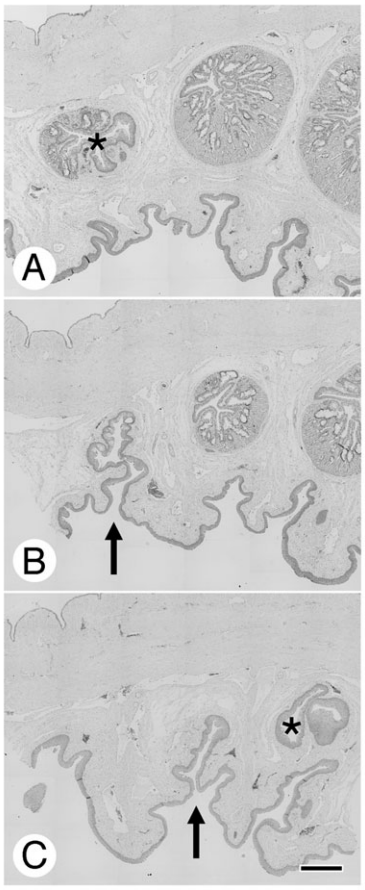

Fig. 4. A series of cross sectional views of the posterior portion of the glandular tissue from the rostral (A) to caudal (C), showing openings of the glandular structures into the lumen of the intestine (arrows). Asterisks indicate central ducts. Scale bar, 500 μm.

system of holocephalan fishes represents primitive traits of the urea-based osmoregulation in cartilaginous fishes. As mentioned above, however, physiological investigation using healthy, captive fish has been considered to be difficult in holocephalan fishes. In one study, *H. colliei* were obtained and maintained in the laboratory (Read, 1971); however, the animals did not feed in captivity and therefore were sampled for plasma and urine compositions within 2 days after capture to ensure that the fish were healthy (Read, 1971). In the present study, most animals began to feed in the holding tank during a 2- to 3-week post-capture period, which provided a recovery period after the stress of capture and transportation. Therefore, this is the first study in which physiological investigations of holocephalan fish have been performed using healthy fish that had been acclimated to their captive environment. The second experiment, however, was performed with unfed fish only 3 days after they were captured, because of practical limitations involved with the husbandry of large numbers of fish. We carefully selected uninjured healthy fish; they swam comfortably in the holding and experimental tanks as indicated by flapping of the well-developed pectoral fins. In the two experiments, plasma parameters in control fish were comparable (Tables 1 and 2), indicating that the fish we used in the second experiment were physiologically in good condition.

The present study clearly demonstrated that elephant fish conduct urea-based osmoregulation as in elasmobranchs and other holocephalan species, although plasma concentrations of ions and urea vary among cartilaginous fish species. In elephant fish maintained in full-strength SW, plasma was nearly iso-osmotic to surrounding SW, and plasma Na^+, Cl^- and urea concentrations were equivalent to those typically reported in elasmobranchs (see Hyodo et al., 2004b). Our results are consistent with values previously reported in *C. milii* (urea, 2.4–2.86%; Cl^-, 1.33% of NaCl) (Dakin, 1931). In elephant fish, urea represents nearly half of the plasma osmolality; therefore, urea contributes greatly to the total osmotic concentration of the body fluids in elephant fish compared to other chimaera species.

Although elephant fish could not tolerate large changes in environmental salinity, they increased or decreased their plasma ion and urea concentrations following transfer to concentrated or diluted SW, respectively. These results indicate that elephant fish have the osmoregulatory ability to adjust their plasma composition when encountering an altered environmental salinity. In the present study, fish survived a gradual increase in environmental salinity to 120% SW, and a rapid increase to 115% SW at least for 3 days. On the other hand, one out of five fish died after gradual dilution to 80% SW; and in a preliminary experiment, fish did not survive a sudden decrease in environmental salinity to 85%. These results imply that elephant fish can osmoregulate in the concentrated SW environment, but they are less adaptable to a low salinity environment. Indeed, they lost a large amount of urea from their blood after transfer to diluted SW in the first experiment (Table 1), suggesting that their ability to retain high levels of urea is less-developed than in elasmobranchs, such as the banded dogfish *Triakis scyllium* (Hyodo et al., 2004b), and the Atlantic stingray *Dasyatis sabina* (Janech et al., 2006). These results correspond well with the ecological reports as described below. In the Port Phillip and Westernport bays, major egg-laying areas for Australian elephant fish, the salinity of the water fluctuates depending on the season. The bay water evaporates during the egg-laying season (summer to autumn), with the level of evaporation exceeding the turnover of oceanic tidal waters. Thus the bay water generally has a higher salinity than the ocean; in both bays, the maximum salinities were 37‰ to 39‰ (Longmore, 1997; Longmore, 2005). In winter and spring, however, the bay water is diluted by run-off from the rivers; minimum salinities recorded in the period 2002 to 2005 for the surface and bottom waters of the Port Phillip Bay were 27‰ and 33.3‰, respectively. From winter to spring, adult elephant fish migrate offshore, but the eggs incubate until hatching in September to October. Hatched eggs have been found in the Barwon River estuary as far upstream as 8 km from the river mouth. Elephant fish osmoregulatory ability may be altered during growth and maturation, and developing and young elephant fish may have good adaptability to a low-

Table 2. *Changes in plasma parameters and rectal gland Na^+/K^+-ATPase activity after rapid transfer to concentrated (115%) seawater (SW)*

	N	Osmolality (mOsm kg^{-1})	Ion concentration (mmol l^{-1})			Na^+/K^+-ATPase (nmol ADP μg^{-1} h^{-1})
			Na^+	Cl^-	Urea	
Day 0 (100% SW)	7	1068.1±4.1 [100%]	292.2±3.7 [100%]	288.4±4.5 [100%]	465.6±8.2 [100%]	3.91±0.38 [100%]
Day 1	5	1174.8±5.2* [110.0%]	337.1±3.4* [115.4%]	327.2±6.0* [113.4%]	499.1±9.2* [107.2%]	4.68±1.07 [119.7%]
Day 2	5	1201.3±7.1* [112.5%]	333.7±3.7* [114.2%]	328.4±4.1* [113.9%]	509.3±8.9* [109.4%]	5.73±0.74 [146.5%]
Day 3	5	1204.6±7.1* [112.8%]	341.4±5.8* [116.9%]	328.8±4.9* [114.0%]	512.1±7.2* [110.0%]	8.18±0.82* [209.2%]

*Significantly different from the Day 0 group at $P<0.01$.
Percent changes from the Day 0 group are shown in square parentheses.

salinity environment. The developmental changes in the osmoregulatory capacity have been described in teleost fishes (see Varsamos et al., 2005). The alteration in osmoregulatory ability is closely related to the ecological features of the species, such as migratory and coastal spawning patterns. An examination of the osmoregulatory ability of developing and young elephant fish is therefore of great interest.

Since there is a substantial concentration gradient for diffusive entry of NaCl into marine cartilaginous fish, they must excrete excess NaCl to maintain their plasma Na^+ and Cl^- concentrations at levels approximately half that of seawater. Elasmobranchs (sharks and rays) have a specialized salt-secreting organ, a rectal gland, from which Na^+ and Cl^- are actively secreted against concentration gradients (Olson, 1999). In elephant fish, however, we could not find a discrete rectal gland. Instead, unique tubular structures were embedded in the wall of the post-valvular intestine. Similar structures have also been found in other holocephalan species; the presence of glandular tissue in chimaeras was first described by Leydig (Leydig, 1851), and later reported in detail (Crofts, 1925). It can be thus generalized that holocephalan fishes have the glandular tissue in the wall of post-valvular intestine (Crofts, 1925; Fange and Fugelli, 1963; Lagios and Stasko-Concannon, 1979). Most probably, the glandular tissue represents a primitive form of the rectal gland in holocephalan fishes. The glandular tissue of elephant fish is well developed compared to that of chimaeras. This difference may be related to the salt-secreting ability of holocephalan species, which may account for the lower plasma NaCl concentration in elephant fish.

From microscopic observations the glandular tissue can be clearly separated into two layers: the outer layer consisting of small tubules, and the inner layer consisting large rectangular cells (Figs 2 and 3). A central duct that is similar to that of elasmobranchs (Olson, 1999) runs longitudinally from the anterior end to the posterior end, where the duct opens to the intestinal lumen. The small tubules in the outer layer consist of a single-layered columnar epithelium which stained intensely with anti-Na^+,K^+-ATPase serum (Figs 2 and 3). The tubules are tightly packed but interspersed with capillaries or sinusoidal vasculatures. These results imply that the abundant small epithelial tubules function as secretory epithelia that excrete Na^+ and Cl^- into the lumen. The large rectangular cells in the inner layer seem to be mucous cells, because these cells were PAS positive (Figs 2 and 3). In a section in which some of the secretory tubules were cut longitudinally, transition from the secretory epithelium to the mucous cells was visible along the same tubule (Fig. 3C,D). The secretory tubules in an individual lobular gland thus drain into the collecting tubules lined by cuboidal mucous cells, and then merge together to form the large central duct. These observations suggest that the fluid secreted from the Na^+,K^+-ATPase-positive epithelia flows into the large lumina of the collecting tubules lined with the mucous cells; the fluid then enters the central duct, and is finally excreted into the lumen of the rectum. At present, the function of the mucous cells is not clear, although they occupy a large mass in the elephant fish glandular structures. From their preponderance in this tissue, it is likely that the mucous cells have an important function in glandular operation: for example, facilitating the excretion of Na^+ and Cl^- by attracting ions (Roberts and Powell, 2003). It is also interesting to note that the collecting and central ducts of the elasmobranch rectal gland are also lined with mucous cells.

In the second experiment, fish were acutely transferred from 100% SW to 115% SW, and sampled on days 0, 1, 2 and 3. Plasma Na^+ and Cl^- concentrations increased rapidly, whereas plasma urea levels increased gradually (Table 2). The Na^+,K^+-ATPase activity increased following the transfer to 115% SW; the increase was statistically significant on day 3. These results demonstrate that elephant fish control plasma urea levels to adjust their plasma osmolality to the increased environmental salinity, and that excretion of NaCl was enhanced by transfer to a high salinity environment, as has been observed in teleost gills and the opercular membrane (for reviews, see Evans, 2002; Hirose et al., 2003). Taken together, the present morphological, histochemical and physiological studies clearly indicate that the glandular tissue embedded in the wall of the post-valvular intestine actually functions as a salt-secreting gland, the rectal gland, in elephant fish. A future study on collection of the secreted fluid will provide a definitive proof

of the salt-secreting function of the tissue. Since the rectal gland of elephant fish is much thicker and more well developed than that of other chimaera species, it is likely that the salt-secreting ability of the gland in elephant fish is superior to that in other chimaeras. This hypothesis is consistent with the observed plasma parameters; elephant fish plasma has lower Na^+ and Cl^- concentrations than other chimaeras, such as, *Chimaera monstrosa* (Fange and Fugelli, 1963). Since elephant fish have evolved a littoral life-style, probably evolving from a common deep-sea ancestor, their enhanced osmoregulatory system may be an adaptation to survive in seawater of varying salinity, which deep-sea species do not encounter.

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