学位論文

Osmoregulation in the developing embryos of oviparous cartilaginous fishes

(卵生軟骨魚類の発育段階における浸透圧調節機構に関する研究)

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Abstract

In a high-salinity and high-osmolality marine environment, cartilaginous fish, which is comprised of two subclasses, the Holocephali (chimaeras) and Elasmobranchii (sharks, skates and rays), have adopted a ureosmotic strategy. They retain a high concentration of urea in their body and maintain the plasma slightly hyperosmotic to the surrounding seawater. It has been shown that in cartilaginous fish urea production is a crucial factor for retaining a high concentration of urea, and that urea is mainly produced via the ornithine urea cycle (OUC) in the liver. However, little is known about the mechanism of urea retention and urea producing organs of the developing embryos in which the adult organs are absent or not fully developed. The central aim of this study is to uncover the adaptation mechanisms to the marine environment in developing embryos of cartilaginous fishes, by using two oviparous species, the elephant fish (Holocephali, *Callorhinchus milii*) and the cloudy catshark (Elasmobranchii, *Scyliorhinus torazame*). Elephant fish is the only cartilaginous fish in which a nearly fully-covered genome database is disclosed, while eggs of the catshark are available throughout the year.

Unlike elasmobranchs, little is known about osmoregulatory mechanisms in holocephalan fishes except that they conduct urea-based osmoregulation, as do elasmobranchs. Therefore, in **chapter 1**, I examined the mRNA expression and activities of the OUC enzymes in the adult holocephalan elephant fish, in order to identify the urea producing organs. For this purpose, I obtained mRNAs encoding carbamoyl phosphate synthetase III (cmCPSIII) and ornithine transcarbamylase (cmOTC), two glutamine synthetases (cmGSs), and two arginases (cmARGs). The two cmGSs were distributionally and functionally separated into two types: brain/liver/kidney-type cmGS1 and muscle-type cmGS2. In addition, two alternatively spliced transcripts with different sizes were found for cmGS1 gene. The longer transcript has a putative mitochondrial targeting signal and was predominantly

expressed in the liver and kidney, suggesting the presence of functional OUC in those organs. High mRNA expression and activities of OUC enzymes were found in the liver, kidney and muscle of adult and hatched fish. These data show that the liver is the major organ for urea biosynthesis in elephant fish, but extra-hepatic tissues such as the kidney and muscle may also contribute to urea production. The molecular and functional characteristics of multiple isoforms of GSs and ARGs are discussed in this chapter.

In **chapter 2-1**, I then investigated the gene expression profiles of OUC enzymes in elephant fish embryos. The most noteworthy finding was that the extra-embryonic yolk sac membrane (YSM) makes an important contribution to the ureosmotic strategy of the early embryonic period. The expression of all OUC enzyme genes was detectable in the embryonic body from at least stage 28, and increased markedly during development to hatching, most probably due to the growth of the liver. During the early developmental period, however, the expression of OUC enzyme genes was not prominent in the embryonic body. Meanwhile, I found that the mRNA expression of OUC enzymes was detected in the YSM; the mRNA expression of cmCPSIII in the YSM was much higher than that in the whole embryonic body during stages 28-31. Significant levels of enzyme activity and the existence of mitochondrial-type cmGS1 transcripts in the YSM support the findings of mRNA expression. I also found that the cmCPSIII transcript is localized in the vascularized mesendodermal inner layer of the YSM. Taken together, these findings suggest for the first time that the YSM is a crucial organ in urea-based osmoregulation, in place of the embryonic liver during the early to mid phase of development in oviparous elephant fish.

To prove that the significant contribution of YSM for urea production is a common phenomenon of oviparous cartilaginous fishes, in **chapter 2-2**, I used cloudy

catshark embryos and investigated the urea production during development. The concentration of urea both in yolk and the embryonic body were maintained at levels similar to the adult plasma concentration throughout development, confirming that catshark embryos also conduct urea-based osmoregulation. Consistent with the findings in elephant fish embryos, the results of quantitative PCR analyses demonstrated that the mRNA expression of catshark stGS, stCPSIII, stOTC and stARG was kept at higher levels in YSM than in the embryo until stage 32. In the YSM, all OUC enzyme mRNAs were predominantly expressed in the highly-vascularized mesendodermal inner layer of YSM. During this period, the highest enzyme activities were also found in the mesendodermal layer. The mRNA abundance of OUC enzymes, in particular the rate-limiting stCPSIII in the YSM, was rapidly decreased after stage 32, while that in the embryonic body was markedly increased. The important contribution of YSM and embryonic body to urea production in the early-middle and late developmental phases, respectively, was also confirmed by the results of OUC enzyme activities. Taken together, the results in this chapter support my idea that the YSM of oviparous cartilaginous fishes contributes to embryonic urea homeostasis before the liver becomes fully functional, in order to cope with high-salinity and high-osmolality external environments.

In **chapter 3**, I attempted to identify the upstream regulatory factor, which may trigger distributional shift of the urea producing organ from the YSM to embryonic liver during the development of oviparous cartilaginous fish. I performed transcriptome analysis on the catshark embryo and YSM, and focused on the two nuclear receptors, glucocorticoid and mineralocorticoid receptors (GR and MR). The full-length coding sequences of GRs and MRs were determined in catshark and elephant fish, and the ligand selectivities and sensitivities of GRs and MRs were examined by an *in vitro* reporter assay. The transactivation activities of cartilaginous fish GRs were

induced by corticosterone more potently than by 1α-hydroxycorticosterone, a major circulating corticosteroid. The administration of corticosterone to the *in vivo* culture system upregulated the stARG mRNA levels in the mesendodermal layer of YSM, while the increase was reversed by co-administration with its inhibitor RU486. The corticosterone treatment also significantly increased stGS mRNA levels in the embryonic body and the ectodermal layer of YSM, while reducing stGS mRNA levels in the mesendodermal layer. Taken together, my findings suggest the importance of glucocorticoids and their receptors in the regulation of urea production in developing embryos of cartilaginous fishes.

In the last part of the thesis, these findings are discussed in more detail from the viewpoint of comparative physiology and endocrinology. I further provide future directions for studying the physiological roles of YSM and the endocrine control of urea homeostasis during the development of oviparous cartilaginous fishes.

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General Introduction

As a critical component of amino acids, proteins and nucleic acids, nitrogen is an essential chemical element for all living organisms. In heterotrophic animals, proteins and other nitrogen-containing molecules are key dietary components, and thus protein metabolism is a vital process for them. However, all animals face the major problem of handling ammonia, the end product of protein catabolism, because of its high toxicity to the nervous systems when it is concentrated in tissues (Cooper and Plum, 1987). Most aquatic animals, including teleosts and larval amphibians are known to be ammonotelic, and relatively ammonia-tolerant; they excrete ammonia directly into the surrounding environment mainly through the gills. Ammonia is highly soluble in water, therefore it is feasible that aquatic animals excrete ammonia directly by diffusion. Meanwhile, most terrestrial vertebrates, including reptiles, birds and mammals, cannot excrete ammonia directly to the environment. They convert ammonia to less toxic nitrogen compounds, either urea or uric acid, for excretion. This is not only for the purpose of reducing toxicity, but also for the reduction of water loss. According to Wright (1995), urea requires about 10 times less volume of water than ammonia for excretion, whereas uric acid is highly insoluble and requires about 50 times less water. Since water availability is limited in terrestrial environments, these two modes of nitrogen excretion, ureotely and uricotely, are advantageous for water conservation. Taken together, the excretory patterns of nitrogenous wastes in extant vertebrates generally differ depending on the water availability. A representative example is anuran amphibians, which mainly excrete ammonia as nitrogenous wastes before metamorphosis (tadpoles), but the excreta are transformed to urea after metamorphosis (Brown et al., 1959). However, this theory is not necessarily applicable to all aquatic and terrestrial animals. Marine cartilaginous fish (sharks, skates, rays and chimaeras), coelacanths, and some teleosts predominantly excrete urea (Pickford and Grant, 1967; Anderson, 2001; Hazon et al., 2003), even though they are

living in an aquatic environment. Furthermore, some terrestrial invertebrates including gastropods, isopods and brachyurans are known to excrete ammonia by ammonia volatilization (Speeg and Campbell, 1968; Wieser et al., 1969; Greenaway and Nakamura, 1991).

In 1929, Smith measured the body fluid composition of ureotelic marine cartilaginous fishes, and found that they control plasma ion concentrations to the level about half that of surrounding seawater (SW), while they retain notably high concentrations of urea (300-450 mM) in the tissues and plasma. He mentioned, for the first time, the biological significance of urea for osmoregulation in marine cartilaginous fishes (Smith, 1936). The accumulation of urea raises their plasma osmolality to slightly hyperosmotic to the surrounding SW, and thereby they do not suffer from dehydration even in the high-osmolarity SW environment. This osmoregulatory strategy is completely different from that of marine teleost fish. Since teleosts maintain their plasma osmolality about one third that of surrounding SW, they lose water because of the large osmotic gradient between the internal and external environments. To overcome the dehydration, marine teleost fishes drink SW constantly and absorb water through the digestive tract, while excess salts are actively excreted mainly through ionocytes in the gills. After Smith (1936), Pickford and Grant (1967) demonstrated that the ureotelic coelacanth also retains similar concentrations of urea in tissues and plasma to maintain internal osmolality at levels nearly iso-osmotic to the surrounding SW. In tetrapods, the crab-eating frog (*Fejervarya cancrivora*) is the only species that accumulates a considerable amount of urea in the body in brackish environments (Gordon et al., 1961). Therefore, urea is an essential molecule for excreting nitrogenous wastes but also functions as an osmolyte for environmental adaptation. Cartilaginous fish, coelacanths and the crab-eating frog are referred to as ureotelic and ureosmotic animals.

Urea-producing mechanims in vertebrates

Urea is synthesized through four potential pathways, three are catabolic (argininolysis, urate degradation and conversion of arginine) and one is anabolic (ornithine urea cycle, OUC) (Walsh and Mommsen, 2001). The argininolysis and final step of the OUC are mediated by arginase (ARG; EC 3.5.3.1), which converts dietary or biosynthesized L-arginine into L-ornithine and urea. This enzyme can be broadly found among the three domains of organisms, bacteria, archaea and eukaryota (Jenkinson et al., 1996), and plays an important role in the urea production of most species. However, in ureotelic vertebrates, the major role of ARG is considered to be the OUC rather than the degradation of dietary arginine (Anderson, 2001). The classical OUC found in ureotelic mammals and amphibians is comprised of the following five enzymes: carbamoyl phosphate synthetase I (CPSI; EC 6.3.4.16), ornithine transcarbamylase (OTC; EC 2.1.3.3), argininosuccinate synthetase (ASS; EC 6.3.4.5), argininosuccinate lyase (ASL; EC 4.3.2.1) and ARG. Three of the OUC enzymes, ASS, ASL and ARG, function in the cytosol, while CPSI and OTC are mitochondrial enzymes. The first step of ammonia fixation is the conversion of ammonia and bicarbonate into carbamoyl phosphate, and the reaction is catalyzed by CPSI. The enzyme activity of CPSI is controlled by its substrate concentration and also by the concentration of positive allosteric effector *N*-acetyl-L-glutamate (Anderson, 1980). In addition, the amount of CPSI is quite low, implying that the reaction mediated by CPSI is a rate-limiting step of the OUC *in vivo*. In contrast, in the OUC of bony fish and cartilaginous fish, ammonia is initially fixed by mitochodrial glutamine synthetase (GS; EC 6.3.1.2), and the piscine-type CPS, CPSIII, catalyzes the subsequent carbamoyl phosphate synthesis by utilizing glutamine as its substrate, rather than ammonia (Fig. I). Therefore, mitochondrial GS is an essential accessory enzyme in the piscine OUC (Anderson and Walsh, 1995). Furthermore, unlike classical OUC,

ARG is known as a mitochondrial enzyme in the piscine OUC.

In ureotelic mammals, complete OUC enzymes are detected only in the liver, whereas some of them are expressed at considerable levels in small intestine and kidney (Morris, 1992). These extra-hepatic OUC enzymes are independent of the OUC and mainly contribute to arginine biosynthetic pathway. Meanwhile, recent studies have revealed that several extra-hepatic tissues of cartilaginous fish, including muscle and digestive tracts, contribute to urea production (Tam et al., 2003; Steele et al., 2005; Kajimura et al., 2006), in addition to the well-established functional OUC in the liver. The capacity of spiny dogfish (*Squalus acanthias*) CPSIII in the muscle was estimated to be about 8- to 23-fold higher than that in the liver, considering its large mass. Thus, the urea-producing system appears to be much more complicated in cartilaginous fish than in other vertebrates, and the extra-hepatic contribution must be taken into account in order to understand systemic urea synthesis comprehensively.

The significance of cartilaginous fish study

The Class Chondrichthyes (cartilaginous fish) is the earliest extant jawed vertebrates (gnathostomes), comprised of two subclasses, Elasmobranchii (sharks, skates, and rays) and Holocephali (chimaeras). It has been generally accepted that the extant species are derived from Mesozoic forms, yet how extant forms relate to the more diverse Paleozoic forms are not fully understood (Grogan et al., 2004). At least, monophyly of the chondrichthyans is supported by the morphological features of many fossils (Maisey, 1984, 1986) and by the molecular phylogenetic analyses of jawed vertebrates (Kikugawa et al., 2004; Inoue et al., 2010).

Cartilaginous fishes currently contain 1,185 extant recognized species (White and Last, 2012), and represent larger size relative to most bony fishes, the average maximum total length among existing sharks being about 1.5 meters (Compagno, 1981).

Unlike bony fishes, about 40% of which inhabit the freshwater (FW) environment (Cohen, 1970), most species of cartilaginous fishes are currently found in marine habitats. Only a few elasmobranchs, for example, the South American river stingrays (Potamotrygonidae) are confined to the FW environment (Rosa et al., 1987). It is assumed that Potamotrygonidae have a full set of OUC enzymes, but they are not functional compared with marine cartilaginous fishes. Their osmoregulatory strategy is identical to "freshwater teleosts", and they cannot survive in a high-salinity environment (over 50% SW) (Gerst and Thorson, 1977; Duncan et al., 2011; Ballantyne and Fraser, 2013). Whereas most littoral species can penetrate into brackish creeks, only a few of them such as large requiem sharks (Carcharhinidae) and sawfishes (Pristidae) are known to be able to adapt to both SW and FW environments. Interestingly, it is reported that the bull shark (*Carcharinus leucas*) retains a considerable amount of urea in the plasma (approximately 150 mM) even when acclimated to an extremely low osmolality environment (3 mOsm l^{-1}), where they need to excrete excess water (Pillans et al., 2005). Meanwhile, all chimaeras are confined to marine environments.

The unique phylogenetic position and ecophysiological features of cartilaginous fishes as mentioned above, have strongly attracted attention of researchers. However, the physiological studies on this class have not progressed much, compared to tetrapods and teleosts, because of difficulties such as the large size, long life span and limited genetic information.

Embryonic development of cartilaginous fish

The reproductive modes of cartilaginous fish are highly diversified, and it has been considered that the various reproductive styles have evolved from oviparity (Wourms, 1977, Compagno, 1990). According to Compagno (1990), the current

reproductive modes can be grouped into two oviparous types; primitive extended oviparity (single oviparity; 42%), retained oviparity (multiple oviparity; 1%), and four viviparous types; yolk sac viviparity (27%), and three derived forms of viviparity (30% in total). The variety of reproductive styles in cartilaginous fishes is of interest for the insight that they might provide into the evolution of viviparity (Kormanik, 1992). In all reproductive modes, the gestation or egg incubation periods are conspicuously long in general. In oviparous species, for example, the embryonic development within egg case is strongly dependent on the envitonmental temperature (Perkins, 1965), and thus several coldwater species, such as the Alaska skate (*Bathyraja parmifera*), have an estimated incubation time of 3.5 years (Hoff, 2008). Also, in viviparous species, a gestation of longer than 3.5 years is proposed for frilled shark (*Chlamydoselachus anguineus*) (Tanaka et al., 1990). This long-term development makes it difficult to collect embryos at different developmental stages and to investigate the development and physiology of embryos.

Viviparous species keep their pups inside the uterus until parturition (Wourms, 1981; Compagno, 1990), whereas maternal support stops just after oviposition in oviparous species (Kormanik, 1992). The external environments of the developing embryos are thus quite different between oviparity and viviparity. Several preceding studies demonstrated the solute composition of uterine fluid in viviparous species (reviewed by Kormanik, 1992). In the early gestational period of spiny dogfish, embryos are bathed in uterine fluids possessing Na^+ and Cl^- concentrations that are intermediate between maternal plasma and SW, with urea concentrations similar to the maternal plasma levels (Evans et al., 1982; Kormanik and Evans, 1986). In the later gestational period, the intrauterine fluid resembles SW, and the embryos obtain the ability to osmoregulate and ionoregulate by themselves (Kormanik and Evans, 1986; Kormanik, 1988). Meanwhile, in the marbled electric ray (*Torpedo marmorata*),

another viviparous species, urea compositions (and presumably also ion concentrations) of the intrauterine fluid are kept nearly identical to that of maternal plasma throughout the gestational period (Mellinger et al., 1986). Taken together, these findings indicate that the viviparous embryos are protected from the high-salinity marine environment, and that embryos have little necessity for body-fluid regulation by themselves during the early developmental period in viviparous species.

Oviparous cartilaginous fishes lay their eggs on the ocean floor. The egg capsules (cases) exhibit diversity in shape and size between species. In contrast to viviparous species, oviparous cartilaginous fish lay eggs within a few days after fertilization. The corners of egg capsules are plugged with egg white jelly, and thus tightly sealed at oviposition. Thereafter, around one third to half way through the developmental period, anterior end(s) of the capsule open. This early opening is called "pre-hatching" or "eclosion" (Ballard et al., 1993; Hamlett and Koob, 1999). After the pre-hatching, SW flows into the capsule, and intracapsular fluid of the egg case is equilibrated with SW. Meanwhile, the composition of intracapsular fluid prior to pre-hatching is still in debate. The preceding studies on several oviparous species showed that egg capsule walls are highly permeable to ions, urea, mannitol, and glucose (Hornsey, 1978; Foulley and Mellinger, 1980; Mellinger et al., 1986). In the big skate (*Raja binoculata*), the intracapsular fluid of the egg case is identical to SW within 24 hours after oviposition, and this continues throughout the development (Kormanik, 1988). On the other hand, Evans (1981) found significant ionic gradients between inside the egg case and surrounding SW in the little skate (*Leucoraja erinacea*).

If the egg case does not form a barrier against ions, the high-salinity and high-osmolality intracapsular fluid would be a harsh environment for the embryos of oviparous species. This is particularly true for the early developmental period, during which the osmoregulatory organs in adults are not fully developed or not formed in the

embryos. However, there is no convincing evidence showing how the developing embryos adapt to the environment. This may be due to the long egg incubation period and to the small size of the embryos, making it difficult to analyze the body fluid and osmoregulatory organs in each developmental stage (Kormanik, 1992). Needham and Needham (1930) showed that the embryonic system (embryo plus yolk) contains a considerable amount of urea in lesser spotted dogfish (*Scyliorhinus canicula*). Read (1968b) confirmed this finding by investigating various sizes of embryos in the big skate, implying that oviparous embryos also conduct urea-based osmoregulation. In addition, Read (1968a) demonstrated that the early stage of big skate embryo (2.5 cm in size) showed OTC and ARG activities, while Steele et al. (2004) reported the CPSIII, OTC, ARG and GS activities exist in 4-month old little skate embryos. Another problem for investigating the development of cartilaginous fish was the lack of a detailed description of developmental stages until recently. Because of this reason, the analyses using embryos have been conducted based on the size of embryos and on the time period after oviposition. Over the past 21 years, a staging manual of the developing embryos has been introduced for cartilaginous fish based on the studies of two sharks, the lesser spotted dogfish and the Port Jackson shark (*Heterodontus portusjacksoni*), a skate, (winter skate, *Leucoraja ocellata*), and a chimaera (elephant fish, *Callorhinchus milii*) (Ballard et al., 1993; Didier et al., 1998; Maxwell et al., 2008; Rodda and Seymour, 2008).

Hormonal controls of osmoregulation in cartilaginous fishes

Although endocrine control is one of the important research topics of the urea-based osmoregulation in cartilaginous fish, the accumulating body of knowledge has mainly focused on the hormones related to salt and water homeostasis. Littoral and euryhaline species are known to reduce plasma osmolality and urea concentrations

when acclimated to low salinity environments (Hazon et al., 2003). In the low salinity environment, osmotic water influx and loss of ions occurs, similar to freshwater teleost fishes. The euryhaline cartilaginous fish therefore excrete excess water through the kidney, while taking up salts probably via the gill ionocytes, in order to maintain their internal ion and water balance. So far, hormonal regulations of water and salt homeostasis have been reported for the following five hormones: arginine vasotocin (AVT), interrenal corticosteroids, renin-angiotensin system (RAS), vasoactive intestinal polypeptide (VIP) and C-type natriuretic peptide (CNP) (Holt and Idler, 1975; Chipkin et al., 1988; Armour et al., 1993; Wells et al., 2002; Hyodo et al., 2004; Anderson et al., 2006; reviewed by Gelsleichter and Evans, 2012). However, no research investigating hormonal regulation of urea production has been conducted in cartilaginous fishes.

Corticosteroid hormones (mineralocorticoids and glucocorticoids) participate in diverse and numerous physiological and developmental events in vertebrates. In tetrapods, including mammals, it is well recognized that representative glucocorticoids, cortisol and corticosterone, are important for glucose homeostasis, immune function and the stress response (Bentley, 1998; Chrousos, 2009), whereas the primary mineralocorticoid, aldosterone, mainly contributes to body fluid homeostasis by controlling sodium and water reabsorption in the kidney (Carpenter et al., 1961; Mulrow et al., 1962; Bentley, 1998). These distinct functions of glucocorticoid and mineralocorticoid are mediated through two nuclear receptors, glucocorticoid and mineralocorticoid receptors (GR and MR), respectively, in mammals (Stolte et al., 2006). In addition, glucocorticoids also have been implicated in the regulation of urea synthesis in mammalian species; the activities of most urea cycle enzymes were increased by administration of glucocorticoids (Gautier et al., 1977; Lamers and Mooren, 1981; Haggerty et al., 1983; Husson et al., 1983; Hazra et al., 2008). The observed upregulation of OUC enzyme activities is most likely caused by direct action

of glucocorticoids on the gene expression of OUC enzymes through GR, since a glucocorticoid responsive element (GRE), which is the binding site of dimerized GR for enhancing or inhibiting the expression of target genes, has been found in promoter regions of mammalian CPSI and ARG (Takiguchi et al., 1988; Christoffels et al., 1998).

Besides the regulation of urea production in mammals, corticosteroids also play important roles in osmoregulation of teleost fishes (Mommsen et al., 1999; McCormik and Bradshaw, 2006). Cortisol controls branchial ion uptake/secretion and water absorption in the intestine, and thereby contributes to ion and water homeostasis via GR and/or MR (Utida et al., 1972; McCormick et al., 2008; Kumai et al., 2012). In elasmobranch species, a unique 1α-hydroxycorticosterone (1α-OHB) has been reported as the principal circulating corticosteroid (Idler and Truscott, 1966; Truscott and Idler, 1968; Kime, 1977), while other corticosteroids also exist in the plasma at low levels. Based on these findings, it is of great interest to know whether corticosteroids contribute to urea-based osmoregulation by controlling urea production in marine cartilaginous fishes.

Aim of this study

The principal goal in this study is to elucidate the osmoregulatory mechanisms of developing embryos of oviparous cartilaginous fish. As I mentioned above, there are no direct evidence demonstrating from when and how cartilaginous fish embryos conduct urea-based osmoregulation. In order to address these questions, I used two oviparous cartilaginous fishes: holocephalan elephant fish and selachian cloudy catshark (*Scyliorhinus torazame*), a close relative of lesser spotted dogfish, as experimental animals in the present study. Elephant fish is the only cartilaginous fish in which a nearly fully-covered genome database is disclosed (Venkatesh et al., 2014), and they are regarded as an important fishery resource in southern Australia and New

Zealand. Elephant fish inhabit continental shelf waters to the depth of at least 200 m; however, they display a clear seasonal migration into bays in southern Australia and New Zealand during the egg-laying season for oviposition. By keeping captured matured females in the spring, I could obtain two fertilized eggs per week from each individual, and finally could use a total of 121 embryos. Given that the developmental table is also available for this species, elephant fish are one of the most appropriate species for developmental study in cartilaginous fishes. The cloudy catshark is another suitable experimental animal for developmental and physiological studies, since the developmental stage of the catshark is able to be identified by using the staging scheme for the closely-related lesser spotted dogfish. The catshark is widely distributed around the coastal area of Japan, and thereby many eggs are available throughout the year. Furthermore, I could use the preliminary genome database of this species by collaborating with Dr. Kuraku of RIKEN Center for Developmental Biology (CDB).

Because of the few available data on urea-producing organs of holocephalan species, in **chapter 1**, I examined the expression and activities of key OUC enzymes, cmGS, cmCPSIII, cmOTC and cmARG in various organs of adult elephant fish, in order to obtain information for the subsequent investigation of urea production in developing embryos. As described in many elasmobranch species, the liver appears to be a primary organ for urea production also in this species. In addition, a considerable contribution of several extra-hepatic tissues to urea synthesis was also found. In **chapter 2**, I confirmed that embryos of oviparous cartilaginous fish conduct urea-based osmoregulation from the early phase of development. The developmental and distributional changes in the mRNA expression of OUC enzymes were investigated in embryos of elephant fish (chapter 2-1) and catshark (chapter 2-2). In **chapter 2-1**, I found, for the first time, that the extra-embryonic yolk sac membrane (YSM) has a functional OUC, and that the YSM contribute to urea production more importantly than

the embryonic body during the early to middle phases of development. Furthermore, distributional shift of the urea-producing site was found from the YSM to embryonic liver during development. In **chapter 2-2**, I showed the important contribution of YSM to urea production also in catshark, as well as the shift of the urea-producing site from the YSM to the embryonic body, and concluded that these are common phenomena to the holocephalan elephant fish and elasmobranch catshark. In the YSM, the mesendodermal layer was the major site of urea production. Considering the upregulation and downregulation of urea production during the shift of the urea-producing site, expression of OUC enzyme mRNAs is most likely controlled by regulatory factors, such as endocrine substances. Therefore, in **chapter 3**, I conducted next generation sequencing on the YSM and the embryonic body to search candidate factors. I found that corticosteroid receptors (CRs: GR and MR) are expressed both in the YSM and the embryonic body. I also revealed that corticosterone induced the upregulation and downregulation of OUC enzyme gene expression *in vivo*.

By integrating the findings in the three chapters, I will discuss the osmoregulatory mechanisms in the developing embryos of oviparous cartilaginous fishes and the significant role of extra-embryonic tissues for various developmental events throughout the vertebrate taxa, and finally provide future perspectives in the general discussion.

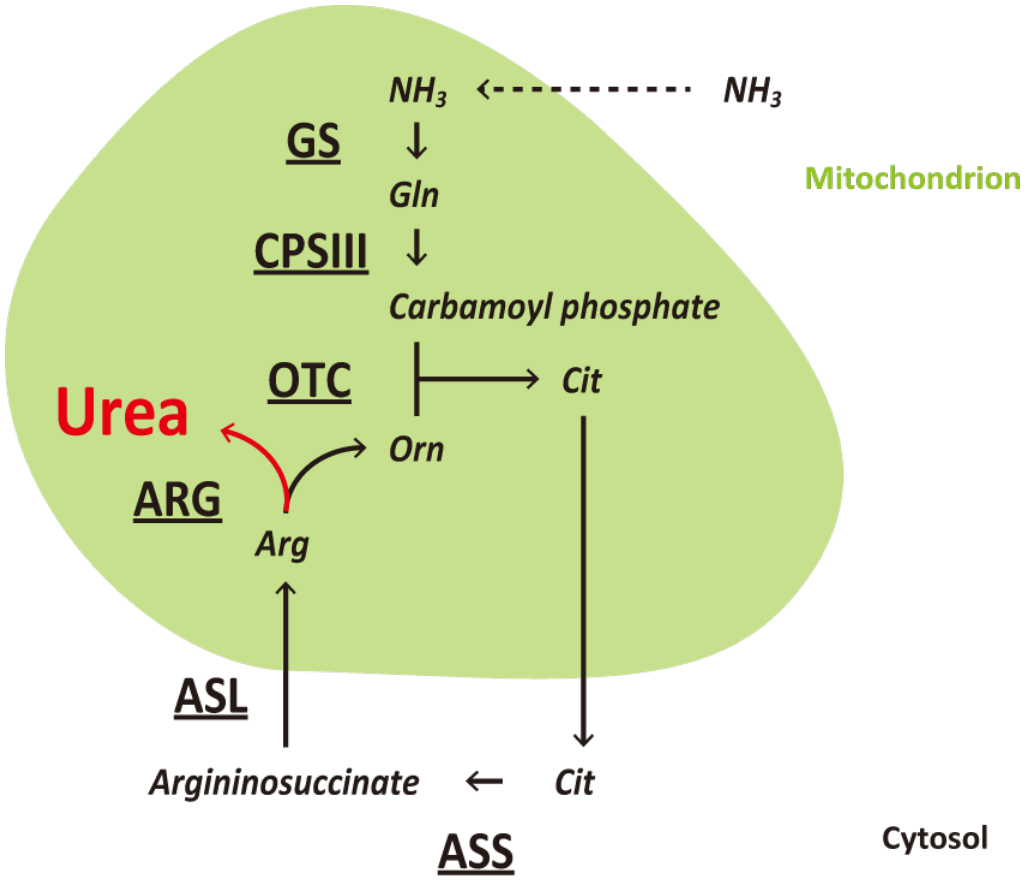

Figure I. Piscine ornithine urea cycle. Cartilaginous fish also have this type of OUC. GS, glutamine synthetase; carbamoyl phosphate synthetase, CPSIII; OTC, ornithine transcarbamylase; ASS, argininosuccinate synthetase; ASL, argininosuccinate lyase; ARG, arginase; Gln, glutamine; Cit, citrulline; Orn, ornithine; Arg, arginine.

Chapter 1

Hepatic and extra-hepatic urea producing organs of adult holocephalan elephant fish (*Callorhinchus milii*)

ABSTRACT

Cartilaginous fish comprise two subclasses, the Holocephali (chimaeras) and Elasmobranchii (sharks, skates and rays). Little is known about osmoregulatory mechanisms in holocephalan fishes except that they conduct urea-based osmoregulation, as do elasmobranchs. In this chapter, I examined the ornithine urea cycle (OUC) enzymes that play a role in urea biosynthesis in the holocephalan elephant fish, *Callorhinchus milii* (cm). I obtained a single mRNA encoding carbamoyl phosphate synthetase III (cmCPSIII) and ornithine transcarbamylase (cmOTC), and two mRNAs encoding glutamine synthetases (cmGSs) and arginases (cmARGs), respectively. The two cmGSs were distributionally and functionally separated into two types: brain/liver/kidney-type cmGS1 and muscle-type cmGS2. Furthermore, two alternatively spliced transcripts with different sizes were found for cmGS1 gene. The longer transcript has a putative mitochondrial targeting signal (MTS) and was predominantly expressed in the liver and kidney. MTS was not found in the short form of cmGS1 and cmGS2. High mRNA expression and enzyme activities were found in the liver, kidney and muscle. Furthermore, in the various tissues examined, mRNA levels of all the enzymes except cmCPSIII were significantly increased after hatching. The data show that the liver is the major organ for urea biosynthesis in elephant fish, but extra-hepatic tissues such as the kidney and muscle may also contribute to urea production. In addition to the role of the extra-hepatic tissues and nitrogen metabolism, the molecular and functional characteristics of multiple isoforms of GSs and ARGs are discussed.

INTRODUCTION

Cartilaginous fish occupy an important position in vertebrate evolution as they are the oldest extant group of jawed vertebrates (the superclass Gnathostomata). The class Chondrichthyes comprises two subclasses, Holocephali (chimaeras) and Elasmobranchii (sharks, skates and rays), which are thought to have diverged in the Silurian about 420 million years ago (Inoue et al., 2010). Marine elasmobranchs overcome the hyperosmotic stress of seawater by retaining urea to maintain their plasma isoosmotic or slightly hyperosmotic to the surrounding seawater (Hazon et al., 1997); they are thus recognized as ureosmotic organisms. To maintain high internal urea levels, *de novo* production of urea as well as reduced urea loss from the kidney and gills are required (Griffith, 1991; reviewed by Walsh and Mommsen, 2001). In contrast to elasmobranchs, little is known about the osmoregulatory mechanisms in holocephalan fishes except that they conduct urea-based osmoregulation as in elasmobranchs (Fänge and Fugelli, 1962; Robertson, 1976; Hyodo et al., 2007).

The liver is considered to be the primary organ for production of urea in elasmobranchs as it expresses the full complement of ornithine-urea cycle (OUC) enzymes, such as the rate-limiting carbamoyl phosphate synthetase III (CPSIII), ornithine transcarbamylase (OTC) and arginase (ARG). In addition, a reduction of environmental salinity concomitantly decreased plasma urea levels and lowered hepatic urea synthesis in a variety of elasmobranch species, further supporting the importance of the liver in maintaining a high plasma urea level (Anderson et al., 2005). Recently, an extra-hepatic contribution to urea production has been reported in several elasmobranch species. In particular, skeletal muscle has been shown to contribute significantly to urea production; in spiny dogfish (*Squalus acanthias*), as CPSIII activity in muscle was 1.6 to 4.7-fold greater than that in the liver (Kajimura et al., 2006).

A growing body of evidence shows that ARG and the accessory enzyme

glutamine synthetase (GS) are encoded by multiple genes in vertebrates (Jenkinson et al., 1996; Murray et al., 2003). In teleost fish, expression of these multiple genes varies between tissues (Walsh et al., 2003; Joerink et al., 2006) and during development (Essex-Fraser et al., 2005), suggesting functional diversity of these multiple genes. However, in holocephalan fishes, there is little information on the molecular basis of urea production and the OUC enzymes.

Recently, the holocephalan elephant fish (or elephant shark, *Callorhinchus milii*), has attracted attention as a model for genome studies of cartilaginous fish (Venkatesh et al., 2005; Venkatesh et al., 2014). The haploid cellular DNA content of the elephant fish was found to be much smaller than other cartilaginous fish genomes (Hinegardner, 1976). Furthermore, the aggregation of large numbers of elephant fish in the shallow waters of southern Australia, Tasmania and New Zealand during the egg-laying season enables the performance of physiological studies on holocephalan fish (Hyodo et al., 2007). In the previous studies, plasma parameters of the elephant fish after transfer to different salinity environments were examined. Plasma osmolality, Na^+, Cl^- and urea concentrations were equivalent to those typically reported in elasmobranchs. Elephant fish have the osmoregulatory ability to adjust their plasma ions, urea and osmolality in altered environmental salinity, which indicates that they are a useful model for the study of the biology of holocephalan fishes (Hyodo et al., 2007; Kakumura et al., 2009).

In this chapter, I cloned cDNAs encoding *Callorhinchus milii* (cm) CPSIII, cmOTC and multiple cmGSs and cmARGs. Expression of the cloned mRNAs, including alternatively spliced variants of the cmGS1 mRNA, was examined in various tissues from fish acclimated in different salinity environments, and fish of peri-hatching stages. The present results revealed the contribution of hepatic and extra-hepatic tissues to nitrogen metabolism in adult elephant fish, and that the multiple GS mRNA

products have distinct functions in separate tissues.

MATERIALS AND METHODS

Fish

Elephant fish, *Callorhinchus milii* (Bory de Saint-Vincent, 1823) of both sexes (total length 75.7±3.2 cm and body mass 1.9±0.2 kg) were collected in Western Port Bay, Victoria, Australia, using recreational fishing equipment, and were transported to Primary Industries Research Victoria, Queenscliff, using a 1,000 L fish transporter. Elephant fish embryos in their egg case were gathered in Western Port Bay by professional divers. The adult fish were kept in a 10,000 L round tank with running seawater (SW) under a natural photoperiod for several days before sampling. Eggs were kept in a 1,000 L tank with running SW, and embryos were sampled in September (before hatching) and October (after hatching). Animals were anaesthetized in 0.1% (w/v) 3-amino benzoic acid ethyl ester (Sigma-Aldrich, St. Louis, MO, USA). Blood samples were obtained from the caudal vasculature with a syringe containing 0.1% ethylenediamine tetraacetic acid (2K-EDTA) and centrifuged at 2,250 *g* for 10 min to obtain plasma. The blood plasma was stored at -20ºC until further analysis. Plasma osmolality and chloride concentration were measured with a vapor pressure osmometer (Wescor 5520, Logan, UT, USA) and a chloride analyzer (C-50AP Chloridemeter, Jokoh, Japan), respectively. Plasma urea concentration was measured using the Wako Urea NB test (Wako Pure Chemical Industries, Japan). After decapitation, brain, gill, heart, liver, kidney, pancreas, spleen, spiral intestine, rectum, rectal gland, skeletal muscle and gonads were dissected out and quickly frozen in liquid nitrogen, and then stored at -80ºC. All animal experiments were conducted according to the Guideline for Care and Use of Animals approved by the committees of the University of Tokyo and Deakin University.

Transfer experiment

In March 2004, the transfer experiment to concentrated (120%) or diluted (80%) SW was performed. Prior to experimentation, fish were kept in a 20,000 L round tank with full-strength SW for at least two weeks, and fed daily with pilchard. A detailed protocol of the transfer experiment has been described previously (Hyodo et al., 2007). In brief, the SW (1069 mOsm kg^{-1}) was gradually concentrated or diluted to 120% (1308 mOsm kg^{-1}) and 80% (824 mOsm kg^{-1}) SW, respectively. On day 4 (1 day after reaching 120% or 80% SW), fish were sampled as described above, and extracted RNA was used for real-time quantitative PCR.

cDNA cloning of OUC enzymes

Cloning of the mRNAs encoding OUC enzymes was performed as previously reported by Kakumura et al. (2009). Total RNA was extracted from the liver, muscle and hypothalamus with guanidium thiocyanate-phenol-chloroform mixture (Isogen, Nippon Gene, Toyama, Japan). After poly-A^+ RNA purification with Oligotex-dT30 (Japan Synthetic Rubber, Tokyo, Japan), adaptor-ligated double-stranded cDNA was synthesized using the SMART cDNA Library Construction Kit (Clontech, Palo Alto, CA, USA). The amino acid sequences of largemouth bass CPSIII (GenBank accession no. **AF006491**), skate OTC (**AF134843**), zebrafish GS (**BC053146**) and rainbow trout ARG (**BK001400**) were used as query sequences to find putative OUC enzyme mRNA sequences in the Elephant Shark Genome Database (http://esharkgenome.imcb.a-star.edu.sg/), and homologous gene fragments were obtained through extensive BLAST searches. Complimentary DNAs of the liver (for cmCPSIII, cmOTC, cmARG2), muscle (for cmGS2 and cmARG1) and hypothalamus (for cmGS1) were used for molecular cloning of mRNAs. Using specific sense and antisense primers complementary to the short sequences obtained from the database,

above-mentioned cDNAs encoding elephant fish OUC enzymes were amplified with Ex-Taq DNA polymerase (TaKaRa, Shiga, Japan). For GS and ARG mRNAs, full-length sequences were obtained by the 3'-RACE method with the SMART cDNA products, and by the 5'RACE method with 5'-Full Race Core Set (TaKaRa). Finally, full-length cDNAs were amplified to confirm the obtained sequences. The amplified products were excised from agarose gels and ligated into a pT7Blue T-Vector (Novagen, Madison, WI, USA). The nucleotide sequences were determined by an automated DNA sequencer (PRISM 3100, Life technologies, Foster City, CA, USA).

Molecular phylogenetic analysis

The deduced amino acid sequences of elephant fish cmCPSIII and the two cmGS and cmARG sequences, respectively, were aligned with those of other animals using ClustalX software (http://www.clustal.org/); sequences were obtained from the DDBJ and Ensembl databases. Molecular phylogenetic trees were constructed by a Bayesian Metropolis coupled Markov chain Monte Carlo method in the MrBayes program (http://mrbayes.csit.fsu.edu/). Four separate Markov chains were run for 500,000 generations, and these were sampled every 100 generations to create a posterior probability distribution of 5,000 trees. The first 1,250 trees were discarded as burn-in before stabilization, and then a 50% majority-rule tree was constructed from subsequent trees. The reliability of the generated trees was shown by posterior probabilities in the Bayesian analysis.

Real-time quantitative PCR assay and RT-PCR

The tissue distribution and expression levels of OUC enzyme mRNAs were determined by real-time quantitative PCR (qPCR) method using a 7900HT Sequence Detection System (Life technologies, Carlsbad, CA, USA), as previously described in

detail (Yamaguchi et al., 2009). For tissue distribution analysis, eight tissues (brain, gill, heart, liver, kidney, spiral intestine, rectal gland and skeletal muscle) from four fish (two males and two females) were examined. Liver and muscle were analyzed from the transfer experiment, while brain, gill, liver, kidney, intestine and skeletal muscle were examined from the peri-hatching fish. Total RNA was extracted as the same method in adult fish. Two μg of total RNA was treated with TURBO DNA-free (Life technologies, Austin, TX, USA) and reverse-transcribed to first-strand cDNA using a High Capacity cDNA Reverse Transcription Kit (Life technologies). To generate a standard curve for mRNA quantification, partial cDNA fragments were obtained with specific primer sets from plasmid containing enzyme cDNAs, and were purified using a QIA quick PCR purification kit (QIAGEN). The cDNAs were then serially diluted, and were used as the known amount of standard cDNAs for absolute quantification for qPCR analyses. The copy numbers of the standard cDNAs were calculated with BioMath Calculators (http://www.promega.com/techserv/tools/biomath/calc01.htm). As internal controls, elephant fish β-actin (cmACTB) mRNA was used for adult fish, whereas elongation factor 1α (cmEF1α) was used for peri-hatching fish, because a study on zebrafish showed that the expression level of the *β-actin* gene is unstable during development, compared to *ef1α* gene (McCurley and Gallard, 2008). Primer sets for qPCR were designed using Primer Express software, and their sequences are shown in Table 1. Real-time qPCR was performed with KAPA SYBR Fast qPCR kit (Kapa Biosystems, Boston, MA, USA). To examine tissue distribution of the long-form and short-form cmGS mRNAs, RT-PCR products were separated on 1% agarose gel, and stained with ethidium bromide.

Analysis of enzyme activities

Enzyme activity assays were conducted on the kidney, liver and muscle,

which were selected from the results of the mRNA expression assay, following the methodology of Kajimura et al. (2006). Tissues were homogenized on ice in 5-10 volumes of homogenization buffer (20 mmol L^{-1} K_2HPO_4, 10 mmol L^{-1} HEPES, 0.5 mmol L^{-1} EDTA, 1 mmol L^{-1} dithiothreitol, 50% glycerol; pH 7.5) using a Brinkman Polytron homogenizer. Homogenates were centrifuged at 8,000 *g* for 20 min at 4°C. The supernatant was used directly for assaying the activity of GS (EC 6.3.1.2), CPSIII (EC 6.3.5.5), OTC (EC 2.1.3.3) and ARG (EC 3.5.3.1). The activities of each enzyme were determined by the colorimetric method via formation of γ-glutamyl-hydroxamate (GS), citrulline (CPSIII and OTC) and urea (ARG), and those were expressed as μmol γ-glutamyl-hydroxamate formed min^{-1} g^{-1}, μmol citrulline formed min^{-1} g^{-1} and μmol urea formed min^{-1} g^{-1}, respectively. The detailed protocols are described in Mommsen and Walsh (1989), Barber and Walsh (1993), and Walsh et al. (1994).

Statistical analysis

Data are represented as means ± SEM throughout the present study. Data from the transfer experiment were analyzed by Steel's non-parametric rank sum test. A Mann-Whitney U test was conducted for comparison between before and after hatching. *P* values less than 0.05 were considered statistically significant.

RESULTS

Identification of urea cycle enzyme cDNAs

I obtained partial OUC enzyme cDNAs by cDNA cloning using specific primers designed on DNA fragments obtained from the elephant fish genome database (Table 1). Since I obtained two distinct cDNAs for cmGS and cmARG, the whole sequences of GS and ARG cDNAs were determined. The nucleotide and amino acid sequences of elephant fish OUC cDNAs were shown to have considerable homology to

other vertebrate OUC enzymes, and were designated as cm*cpsIII* (GenBank accession no. **AB603761**), cm*otc* (**AB622984**), cm*gs1* (**AB622985**), cm*gs2* (**AB622986**), cm*arg1* (**AB622988**) and cm*arg2* (**AB622987**), respectively.

The partial cDNA encoding cmCPSIII contained 4092 nt encoding 1364 amino acid residues, and exhibited high homology to CPSIII of other species (e.g. 90% amino acid identity to spiny dogfish CPSIII). The partial cmCPSIII contained glutaminase and synthetase domains including conserved histidine and cysteine residues. The cysteine residues are essential for N-acetylglutamate-dependent activity and are characteristic of CPSIII but not CPSII (Hong et al., 1994); this confirms the cloning of cmCPSIII cDNA. Molecular phylogenetic analysis further confirmed that the obtained cmCPS belonged to the clade of CPSI and CPSIII but not that of CPSII. The cmCPS was closely related to CPSIII of other fishes and of spiny dogfish rather than orthologous CPSI of terrestrial vertebrates (Fig. 1). The partial cmOTC cDNA contained 824 nt encoding 274 amino acid residues, and had high amino acid identity to OTCs of skate (83.5%), Atlantic salmon (73.2%) and African clawed frog (*Xenopus laevis*) (74.5%). Putative binding sites for two OTC substrates, carbamoyl phosphate and ornithine, were completely conserved in the partial cmOTC (Kraus et al., 1985).

The molecular phylogenetic analysis of known vertebrate ARGs displayed two large clusters consisting of ARG1 and ARG2 (Fig. 2). Two ARG sequences obtained from elephant fish were classified into the ARG1 and ARG2 groups, respectively. The cmARG2 mRNA had a putative ORF of 1065 nt encoding 355 amino acid residues and contained the conserved amino acid residues important for catalysis and stability of the binuclear metal center (Cavalli et al., 1994; Kanyo et al., 1996). *In silico* analysis of cmARG2 with the MitoProt software (Claros and Vincens, 1996) predicted the N-terminal 42 amino acid residues as a mitochondrial targeting signal (MTS) to direct transport of the protein to the mitochondria (probability 0.99).

The conserved amino acid residues were also found when cmARG1 mRNA was translated. However, cmARG1 mRNA exhibited unusual characteristics; expression of cmARG1 mRNA with poly-A^+ tail was confirmed in various tissues but the translated sequence contained multiple termination codons regardless of the reading frame.

The molecular phylogenetic analysis of known vertebrate GSs revealed that vertebrate GSs were classified into two groups (Fig. 3). Most of the known GSs including all mammalian, avian, elasmobranch and most teleost GSs were included in the major group (GS1), while clawd frog and three teleost GSs were grouped into the second clade (GS2). The two GSs cloned from elephant fish were separately classified into GS1 and GS2 groups, respectively. Both amino acid sequences of cmGS1 and cmGS2 contained the highly conserved glutamate binding site (Gill and Eisenberg, 2001).

The 5'-RACE of GS1 mRNA using kidney cDNA showed two transcripts of different sizes (Fig. 4A). The shorter transcript, designated as cmGS1Short mRNA, contained a putative ORF of 1110 nt encoding 370 amino acid residues. The longer transcript (cmGS1Long mRNA) had the same sequence except for the insertion of a 99 nt sequence at the 5' region. The insertion position was 8 or 9 nt prior to the ATG start codon of the short form. This insertion resulted in the occurrence of additional ATG start sites at 27 and 90 nucleotides prior to the start codon of the cmGS1Short mRNA. Therefore, the cmGS1Long mRNA contained a putative ORF of 400 amino acid residues. These results indicate that the two different transcripts are derived from the same cmGS1 gene by an alternative splicing of 5' exons. In the short form, the second exon is most likely absent (Fig. 4A). *In silico* analysis of deduced cmGS1 proteins with the MitoProt software predicted the N-terminal 30 amino acid residues of the long form as an MTS (probability 0.90). On the other hand, the N-terminal

sequence of the cmGS1Short, as well as the 9 amino acid extended N-terminal sequence using the second ATG site in the longer transcript, was not predicted (or low possibility) to contain an MTS.

The full length sequence of cmGS2 mRNA was determined from muscle cDNA. Unlike cmGS1, only a single transcript was found for the cmGS2 gene, and encoded a putative ORF of 373 amino acid residues. *In silico* analysis with the MitoProt software revealed that the cmGS2 does not have an MTS in the N-terminal region.

Tissue distribution of mRNAs and enzyme activities in adult fish

The tissue distribution of mRNAs encoding OUC enzymes was examined by real-time qPCR in four adult elephant fish. Messenger RNA expression of each enzyme was high in the liver. In particular, mean values of cmCPSIII and cmOTC mRNAs in the liver were at levels 24-fold and 7-fold higher than those in the muscle and kidney, respectively (Figs. 5A and B). Among extra-hepatic tissues, relatively high expression of OUC enzyme mRNAs was seen in the muscle (Fig. 5). The kidney also expressed cmGS1, cmOTC and cmARG mRNAs. Expression of cmGS1 mRNA in the brain and kidney was higher than that in the liver (Figs. 5B and C). Interestingly, the two cmGS mRNAs showed complementary expression patterns; cmGS1 gene was expressed in the brain, liver and kidney, while cmGS2 gene was expressed in the gill, heart, rectal gland and muscle (Fig. 5C).

Since the cmGS1 gene showed two transcripts of different sizes, distribution of cmGS1Long and cmGS1Short mRNAs was examined by RT-PCR in tissues in which high expression of cmGS mRNAs was observed. Consistent with the results of qPCR, expression of cmGS1 mRNA was higher in the brain and kidney than the liver (Fig. 4B). The long form with the MTS was predominant in the liver and kidney,

whereas high expression of the short form was observed in the brain. RT-PCR of the 5' portion of cmGS2 mRNA displayed only one band with the highest signal in the muscle, confirming the results of cloning and qPCR analyses (Fig. 4B). These results were consistent among the four elephant fish specimens examined.

To confirm the results of the mRNA expression, enzyme activities were measured for CPSIII, OTC, ARG and GS in the liver, kidney and muscle, where high expression of the enzyme mRNA was found. Enzyme activity in the liver and kidney coincided well with the mRNA expression data (Fig. 6). The highest activities for CPSIII, OTC and ARG were observed in the liver, while the kidney also showed high activities of these enzymes including GS. Meanwhile, ARG and GS activity in the muscle were inconsistent with the mRNA results. Specifically, GS activity was quite low in the muscle (Fig. 6C), although considerably high mRNA expression of cmGS2 was detected in the muscle (Figs. 4B and 5C). Therefore, the activities of OUC enzymes reflected the results of mRNA expression analysis, except for the newly determined cmGS2 and for muscle ARG activity.

Transfer experiment

The expression of OUC enzyme mRNAs in the liver tended to decrease in fish acclimated to 80% SW, and the difference for cmOTC mRNA was statistically significant (Table 2). Consistent with the results of tissue distribution analysis, expression levels of cmGS1, cmCPSIII, cmOTC and cmARG2 mRNAs in the liver were much higher than those in the muscle. Expression of cmARG2 mRNA in the muscle was significantly increased in the 120% SW.

Messenger RNA expression before and after hatching

Plasma osmolality, urea and Cl^- concentrations of embryos were equivalent to

those of adult fish (Table 3). After hatching, no apparent change was observed in those plasma parameters. The tissue distribution of OUC enzyme mRNAs in embryos was similar to adult fish, and the mRNAs encoding cmCPSIII, cmOTC and cmARG2 showed the highest expression in the liver (Figs. 7C, D and E). After hatching, upregulation of mRNAs encoding cmGS1 and 2, cmOTC and cmARG2 were observed compared to embryos (Figs. 7A, B, D and E). No change was observed in CPSIII mRNA levels between embryos and hatched fish (Fig. 7C).

DISCUSSION

This is the first report of the sequence of OUC enzyme mRNAs from a holocephalan fish, including the rate-limiting enzyme, CPSIII, and the accessory enzyme, GS. I found that the elephant fish has two separate GS (cmGS1 and cmGS2) and ARG (cmARG1 and cmARG2) genes. Furthermore, alternatively spliced variants were found for cmGS1 mRNA. Expression of multiple GS and ARG transcripts varied among tissues, suggesting that nitrogen metabolism is intricately regulated in the elephant fish. Quantitative analyses of mRNA expression and enzyme activities revealed that the major organ for urea production in elephant fish is the liver, as has been documented in elasmobranchs (Anderson, 1995, 2001; Walsh and Mommsen, 2001). However, a considerable contribution of extra-hepatic organs including muscle and kidney was also demonstrated.

Multiple transcripts encoding elephant fish GS

The major roles of vertebrate GSs, which synthesize L-glutamine from L-glutamate and ammonia, are considered to be the recycling of the neurotransmitter glutamate, and for ammonia detoxification (Felipo and Butterworth, 2002). In addition, in ureotelic or ureosmotic fish such as marine cartilaginous fishes, glutamine

is essential for urea production (Webb and Brown, 1980). Glutamine, rather than ammonia, serves as the substrate for carbamoyl phosphate formation catalyzed by CPSIII, which is a component of the fish OUC. The ammonia fixation by GS and most of the following OUC steps for urea production occur in the mitochondria of the elasmobranch liver, while GS that is not related to urea synthesis is located in the cytosol (Anderson, 2001). Indeed, in the elephant fish, I found two separate transcripts from the cmGS1 gene. The cmGS1Long mRNA with a predicted MTS was predominant in the liver and kidney, while the cmGS1Short mRNA lacking an MTS was mainly expressed in the brain (Fig. 4B). These results correlated well with the isozyme localization that has been reported in cartilaginous fish: the larger isozyme in the mitochondria of liver and kidney, and the smaller cytosolic isozyme in the brain, spleen and several other tissues (Ritter et al., 1987; Smith et al., 1987; Laud and Campbell 1994).

The present study also showed that intracellular localization of GS isozymes is controlled by an alternative splicing event. In the cmGS1Long mRNA, an additional exon is inserted prior to the ATG start codon found in the short form (Fig. 4A). This additional exon resulted in the occurrence of a new start codon generating proteins 30 amino acids longer than the cmGS1Short mRNA, with a predicted MTS in the extended N-terminal region. The alternative splicing of GS mRNA in the liver and brain (spinal cord) has also been reported in spiny dogfish (Matthews et al., 2005). Transfection of a chimeric EGFP construct revealed that N-terminal sequences of alternatively spliced GS mRNAs show a mitochondrial or cytosolic distribution. Therefore, it is most probable that alternative splicing is the common mechanism in cartilaginous fish to generate tissue-specific subcellular localization of GS isozymes.

In the present study, I found a second GS gene called cm*gs2*. Most of the known vertebrate GSs were included in the GS1 group, while two GSs from African

clawd frog, the second type GS from Japanese pufferfish (*Takifugu rubripes*) and the gill-type GS of gulf toadfish (*Opsanus beta*) were grouped with cmGS2 into the second clade. It has been considered that GS isozymes observed in different tissues are products from a single GS1 gene in elasmobranchs (Laud and Campbell, 1994; Matthews et al., 2005). Therefore, this is the first report of multiple GS genes in a cartilaginous fish. The cmGS1 and cmGS2 mRNAs showed complementary tissue expression patterns, suggesting that GS function is regulated more intricately than previously thought. Unlike cmGS1, cmGS2 mRNA does not have an MTS, which is consistent with the second-type of GS from Japanese pufferfish and the gill-type GS of gulf toadfish (Walsh et al., 2003). Further investigation is necessary to clarify whether elasmobranchs also have the GS2-type gene or not.

Expression of cmGS2 mRNA was predominantly observed in the skeletal muscle, with lower levels observed in the gill, heart and rectal gland; these tissues did not express cmGS1 (Fig. 5C). However, I could only detect low levels of GS activity in the muscle (Fig. 6C), indicating that the translation product of cmGS2 mRNA does not account for GS enzyme activity. A similar situation was found in the toadfish, in which the gill-type GS mRNA was expressed only in the gill, but GS activity in the gill was much lower than in the liver, brain, kidney and stomach (Walsh et al., 2003). Therefore, cmGS2 and the gill-type GS of the toadfish may not contribute to GS activity. Alternatively, conventional methods for GS activity measurement may not be applicable to the second type of GS. Expression of cmGS2 mRNA in the muscle was markedly increased after hatching, implying that the cmGS2 gene product probably has an important role in muscle.

Multiple transcripts encoding elephant fish ARG

It is well-known in ureotelic tetrapods and in teleosts that the ARG has two

isozymic forms encoded by separate genes, *arg1* and *arg2* (Grody et al., 1989; Wright et al., 2004; Joerink et al., 2006). The ARG1 gene is considered to have evolved from an ancestral ARG2 gene, and the function of ARG2 seems to be replaced by that of ARG1 in tetrapods (Srivastava and Ratha, 2010). The finding of two separate ARG genes in cartilaginous fish indicates that the duplication of ancestral ARG gene occurred before the divergence between cartilaginous fish and bony fish.

In elephant fish, cmARG2 is the principal ARG enzyme in the OUC. The deduced cmARG2 amino acid sequence contains a predicted MTS at the N-terminal region, and the mRNA was highly expressed in the liver (Fig. 5D); high ARG activity was also detected in the liver (Fig. 6D). These results are consistent with the elasmobranch OUC model in which ornithine and urea formation catalyzed by ARG occurs in the mitochondria (Anderson, 2001). Unlike cmARG2, the cmARG1 gene seems to have lost its function, since multiple termination codons were found in every reading frame. It is of interest to know whether the ARG1 gene is functional in other cartilaginous fish species.

Contribution of extra-hepatic organs in nitrogen metabolism

The predominant mRNA expression and enzyme activity of the cmCPSIII, cmOTC and cmARG were found in the liver. The liver produces the long form of cmGS1 with MTS, indicating that the liver is the crucial organ for urea production. Another holocephalan, the spotted ratfish (*Hydrolagus colliei*) also exhibited all the OUC enzyme activities in the liver (Read, 1967).

In addition to the liver, the mRNA expression and the activities of the OUC enzymes were detected in other organs, which indicate that extra-hepatic organs contribute to some extent to urea production in elephant fish. In fact, the contribution of extra-hepatic organs to urea production has been recently reported in elasmobranchs.

Kajimura et al. (2006) demonstrated that CPSIII activity was quite high in the muscle and intestine of spiny dogfish. They found approximately an 8 to 23-fold greater CPSIII capacity in the muscle compared to the liver, suggesting that the muscle may be the main site for urea synthesis in the dogfish. A recent study found that CPSIII mRNA levels were markedly reduced in the muscle, but not the liver, when Japanese banded houndshark (*Triakis scyllium*), were acclimated to a low salinity (Yamaguchi et al., unpublished data). I also detected relatively high CPSIII activity (Fig. 6A) in the muscle of elephant fish. When I estimate the weight of muscle as 50% of the whole body weight (Kajimura et al., 2006), the capacity of CPSIII enzyme in the muscle is 4-fold greater than that in the liver. Although enzyme activities of GS, OTC and ARG in the muscle were considerably lower compared to those in the liver (Figs. 6B, C and D), muscle may make relatively important contribution to urea production and retention also in elephant fish.

After hatching, up-regulation of cmGS1, cmGS2, cmOTC and cmARG2 mRNA levels were observed in various tissues including brain, liver, intestine, gill, kidney and muscle (Figs. 7A, B, D and E). On the other hand, no change was found in cmCPSIII mRNA levels. This result correlated well with the plasma urea data; embryos already have a high plasma urea concentration equivalent to newly hatched and adult fish (Table 3). When newly hatched fish start to swim after hatching, various metabolic processes, including nitrogen metabolism, are most likely up-regulated. The increase in GS mRNA levels in various tissues may be important for the recycling of glutamate, ammonia detoxification and retention of the nitrogen source.

The most striking observation for extra-hepatic urea production was the high activities of OUC enzymes in the kidney (Fig. 6). High GS1 mRNA expression was also found in the kidney (Fig. 5C). Previously, high renal GS activity had been observed in several species of elasmobranchs (Webb and Brown, 1980; Chamberlin and

Ballantyne, 1992; Kajimura et al., 2006). In spiny dogfish, King and Goldstein (1983) demonstrated that the concentration of glutamine is relatively high in the kidney compared with the blood, suggesting a high renal capacity for glutamine synthesis. The elephant fish kidney produces the long form of cmGS1 with the MTS, and this result is consistent with previous studies in which the longer GS isozyme was found in the mitochondria of liver and kidney in cartilaginous fish (Smith et al., 1987). Taken together with high OUC enzyme activities, these results imply that the elephant fish kidney converts ammonia into glutamine and/or urea. Recently, Nakada et al. (2010) isolated Rh protein from the kidney of banded houndshark and localized it in the proximal and intermediate segments in the sinus zone of the houndshark nephron. Further studies on the localization of GS and OUC enzymes in the nephron are required, but the kidney is likely to convert absorbed ammonia from the plasma or filtered urine into urea (see General discussion). Reabsorption of urea is a well-known function of the cartilaginous fish kidney in order to retain urea for urea-based osmoregulation (Yamaguchi et al., 2009). The conversion of ammonia or ammonium into glutamine and/or urea is probably another important function of the kidney for urea retention.

Table 1. Primer sets used in the present study

Gene name	Primer sets for real-time qPCR assay (5' to 3')		Primer sets for molecular cloning (5' to 3')	
cm*cpsIII*	Sense:	GGCTGAAGGTTTCCAAAATCC	Sense:	GGATTGTTAGTTCAAGATTACAGC
	Antisense:	AACTCTCCAGCCTGACCAATTG	Antisense:	CCGATAATGATACAGACTGGT
cm*otc*	Sense:	CCTAAGGGTTTTGAACCGAAGA	Sense:	ACACCTCCTCACACTCAAAGA
	Antisense:	TTCGTTCCACACTGTTTTGACAA	Antisense:	CTGCATCATCTACTTCTTCTTGT
cm*gs1*	Sense:	GCTGACTGGATTGCACGAAA	Sense:	CTTTGATGGCTCCAGCACAT
	Antisense:	GGCCCCACGGTTTGCT	Antisense:	GGTGCTGAAGTTGGTGTGA
cm*gs2*	Sense:	GGGCTGAGCTGACAATTTTGA	Sense:	CCAAACTCAGCAAAACGTTG
	Antisense:	TGCATTCACTTTACTAACAGACTGACA	Antisense:	GAGCTTCTGTGACAGCATAAG
cm*arg1*	Sense:	ATAGGAGATACCCAAAAACAAATATGC	Sense:	GGGCGGCAAATCAGAAGTTATCTC
	Antisense:	CGACTGACATAGAGACATGATATATACTTCA	Antisense:	TCCATGCATGTTGCCAGTAGTACTC
cm*arg2*	Sense:	GAAATACACAGCAGCAACCTGATT	Sense:	TTTGCTGGAGAGGCTGTC
	Antisense:	GATGGTGATGTAAGAGGTGTGTTAACA	Antisense:	GAGGCAGTGGTAATATCGATTG
cm*actb*	Sense:	ACATGGAAAAAATCTGGCATCA		
	Antisense:	TGTGAGCAGGACTGGGTGTTC		
cm*ef1a*	Sense:	ACATGCTGGAAGAAAGCGTG		
	Antisense:	TCAGCAGTGTCAGACCGGA		

Transcript name	Primer sets for tissue distribution analysis of cmGS mRNAs (5' to 3')		Amplicon size (bp)
cmGS1Short / Long	Sense:	ACTGCGCGAGAACAGAGACC	430 / 529
	Antisense:	CCATCACCTTATTACACGTACGTCTGTAG	
cmGS2	Sense:	GCAGGAGAGTGGCAGAGAGC	408
	Antisense:	CCCTGAACATACTGACCGGGA	

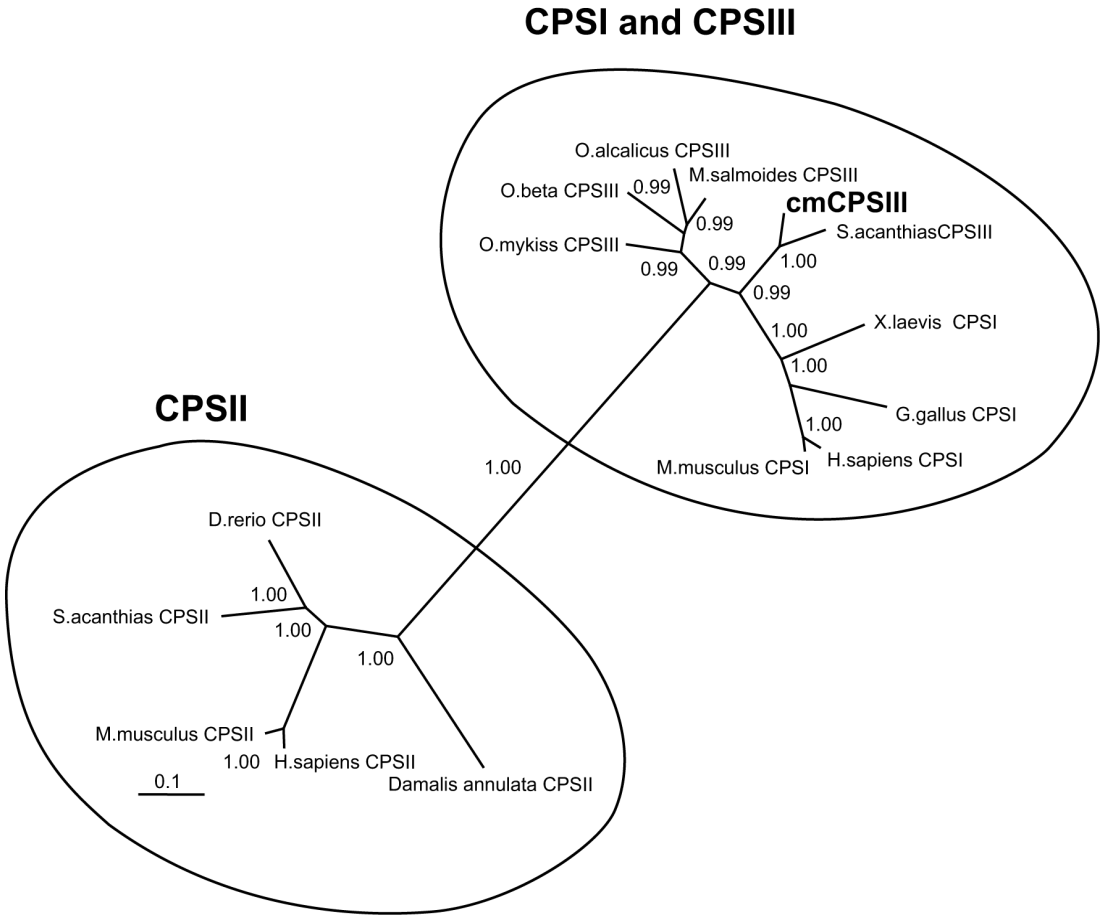

Figure 1. Molecular phylogenetic tree of vertebrate CPS proteins. Numbers at branch nodes represent Bayesian posterior probabilities. GenBank accession numbers are as follows: *Homo sapiens* CPSI, **BC140943**; CPSII, **BC065510**; *Mus musculus* CPSI, **BC126969**; CPSII, **BC137856**; *Gallus gallus* CPSI, **AB159266**; *Xenopus laevis* CPSI, **DQ179105**; *Damalis annulata* CPSII, **EF650404**; *Danio rerio* CPSII, **AY880246***; Micropterus salmoides* CPSIII, **AF006491**; *Oreochromis alcalicus* CPSIII, **AF119250**; *Opsanus beta* CPSIII, **AF169248**; *Oncorhyncus mykiss* CPSIII, **U65893**; *Squalus acanthias* CPSII, **U18868**; CPSIII, **U19771**.

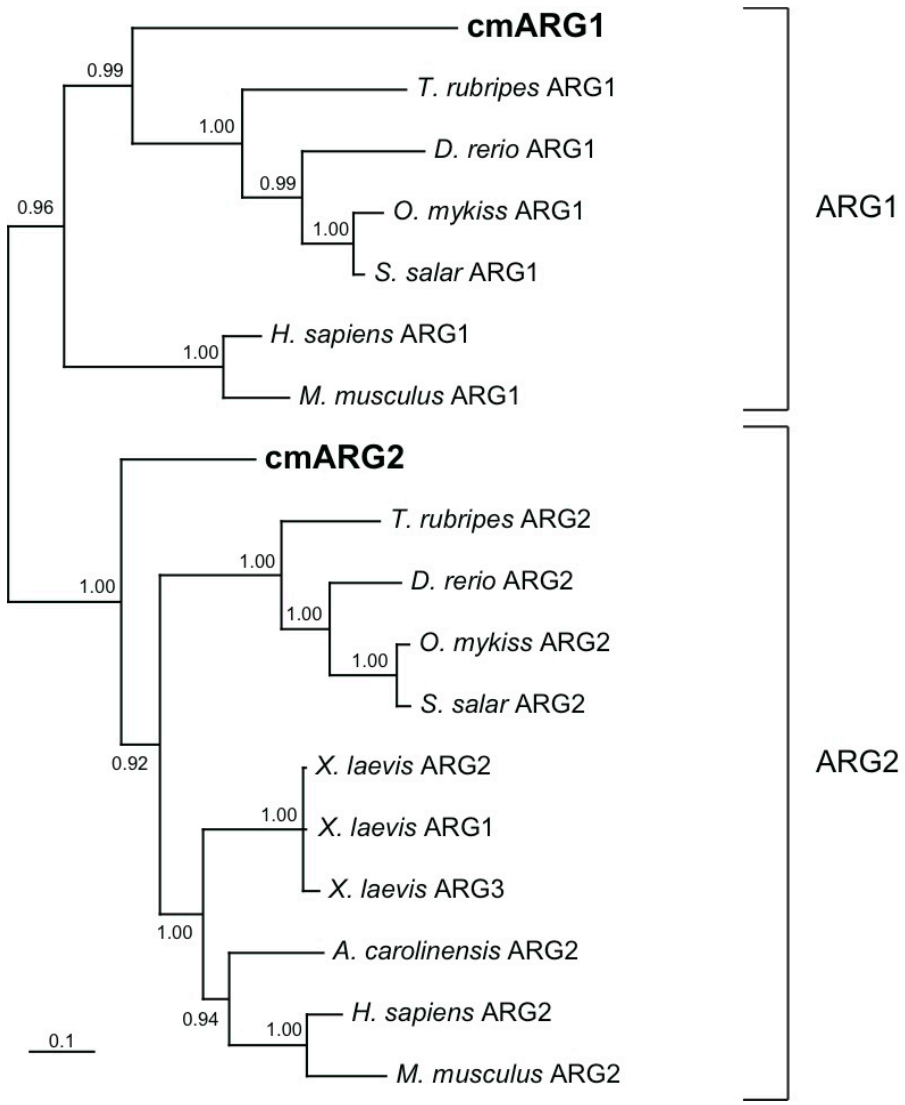

Figure 2. Molecular phylogenetic tree of vertebrate ARG proteins. Numbers at branch nodes represent Bayesian posterior probabilities. *Bombyx mori* ARG sequence (GenBank accession no. **AB564725**) was used as the outgroup. Accession nos. are as follows: *Homo sapiens* ARG1, **DQ892039**; ARG2, **D86724**; *Mus musculus* ARG1, **BC013341**; ARG2, **BC023349**; *Oncorhynchus mykiss* ARG1, **BK001403**; ARG2, **BK001400**; *Salmo salar* ARG1, **BT049081**; ARG2 **BT058927**; *Xenopus laevis* ARG1, **U08406**; ARG2, **U08407**; ARG3, **U08408**. *Anolis carolinensis* ARG2 (Ensembl: ENSACAG 00000005722), *Danio rerio* ARG1 (*arg1*) (ENSDARG 00000071703), *Danio rerio* ARG2 (*arg2*) (ENSDARG 00000039269), *Takifugu rubripes* ARG1 (ENSTRUG 00000002189) and ARG2 (ENSTRUG 00000003963) are sequences from the ensembl database.

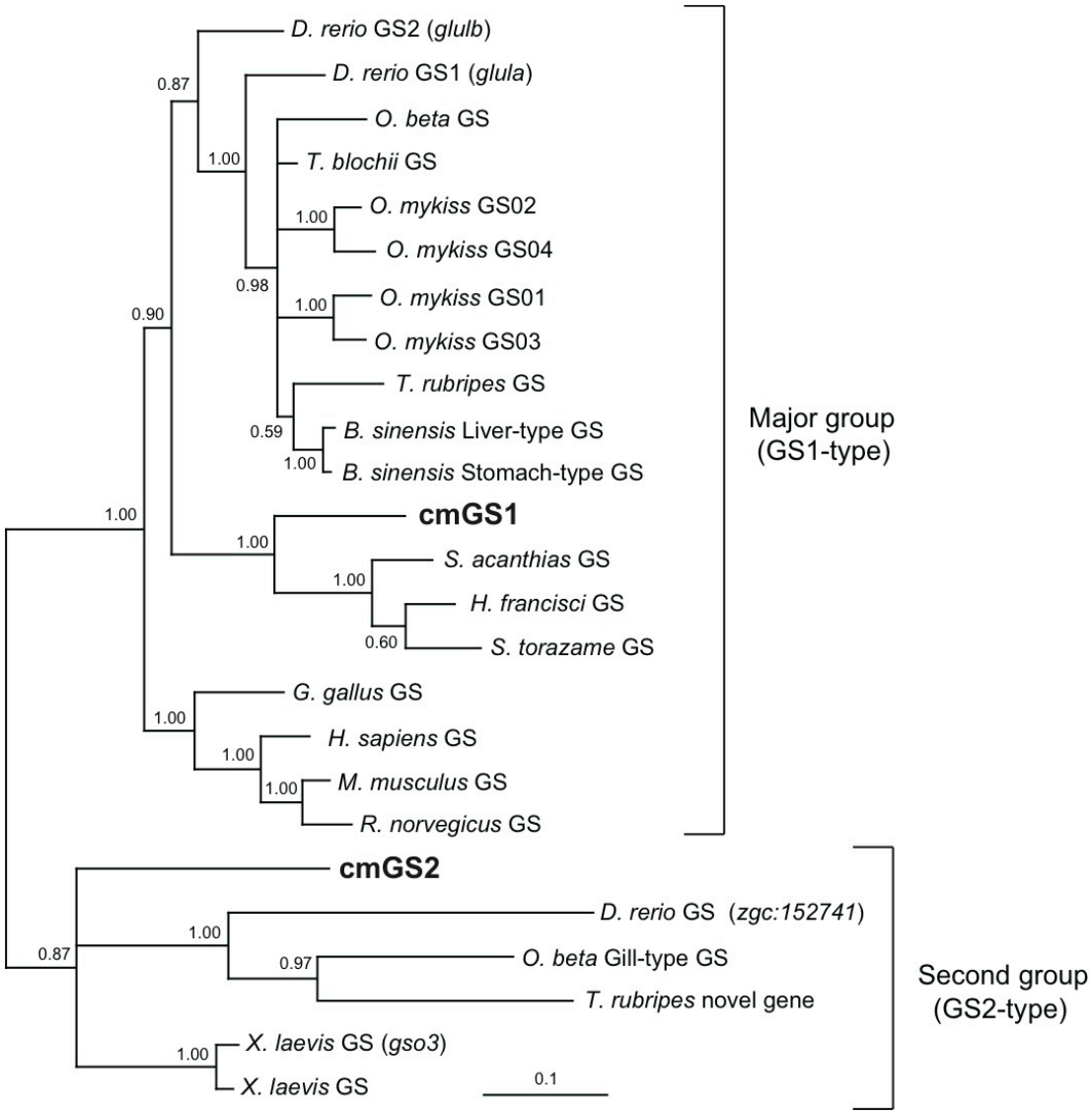

Figure 3. Molecular phylogenetic tree of vertebrate GS proteins. Numbers at branch nodes represent Bayesian posterior probabilities. *Enchytraeus japonensis* GS (GenBank accession no. AB109095) sequence was used as the outgroup. Accession nos. are as follows: *Bostrichthyes sinensis* liver-type GS, **AY071837**; stomach-type GS, **AY071838**; *Gallus gallus* GS, **S45408**; *Heterodontus francisci* GS, **AF118104**; *Homo sapiens* GS, **Y00387**; *Mus musculus* GS, **P15105**; *Oncorhynchus mykiss* GS01, **AF390021**; GS02, **AF390022**; GS03, **AF390023**; GS04, **AF390024**; *Opsanus beta* GS, **AF118103**; gill-type GS, **AF532312**; *Rattus norvegicus* GS, **NM_017073**; *Scyliorhinus torazame* GS, **AF306642**; *Squalus acanthias* GS, **U04617**; *Trachinotus blochii* GS, **GU001782**; *Xenopus laevis* GS, **D50062**; GS (*gso3*), **BK000049**. *Danio rerio* GS1 (*glula*) (ENSDARG 00000017339), GS2 (*glulb*) (ENSDARG 00000069054), uncharacterized GS (*zgc*:152741) (ENSDARG 00000013227), *Takifugu rubripes* GS (ENSTRUG 00000001238) and novel gene (ENSTRUG 00000002542) are sequences from the ensembl database.

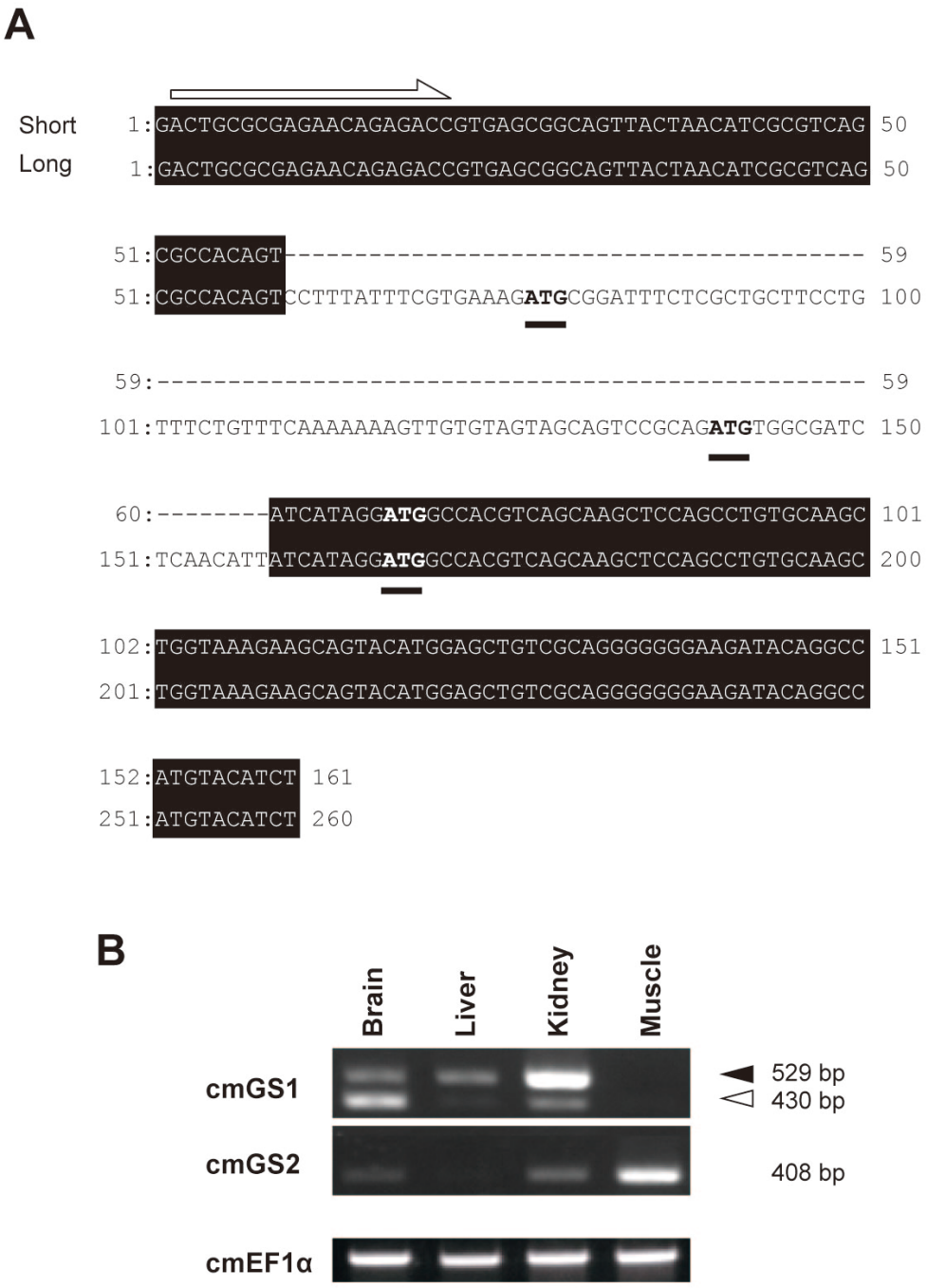

Figure 4. (A) 5' portion of cmGS1Long and cmGS1Short mRNA sequences. Identical nucleotides are indicated in black. Underlined nucleotides (shown in boldface) indicate possible start codons. Arrow indicates the common sense primer used for RT-PCR. (B) Tissue distribution of mitochondrial and cytosolic forms of cmGS mRNAs (cmGS1Long, cmGS1Short and cmGS2). Black and white arrowheads indicate cmGS1Long and cmGS1Short mRNAs, respectively.

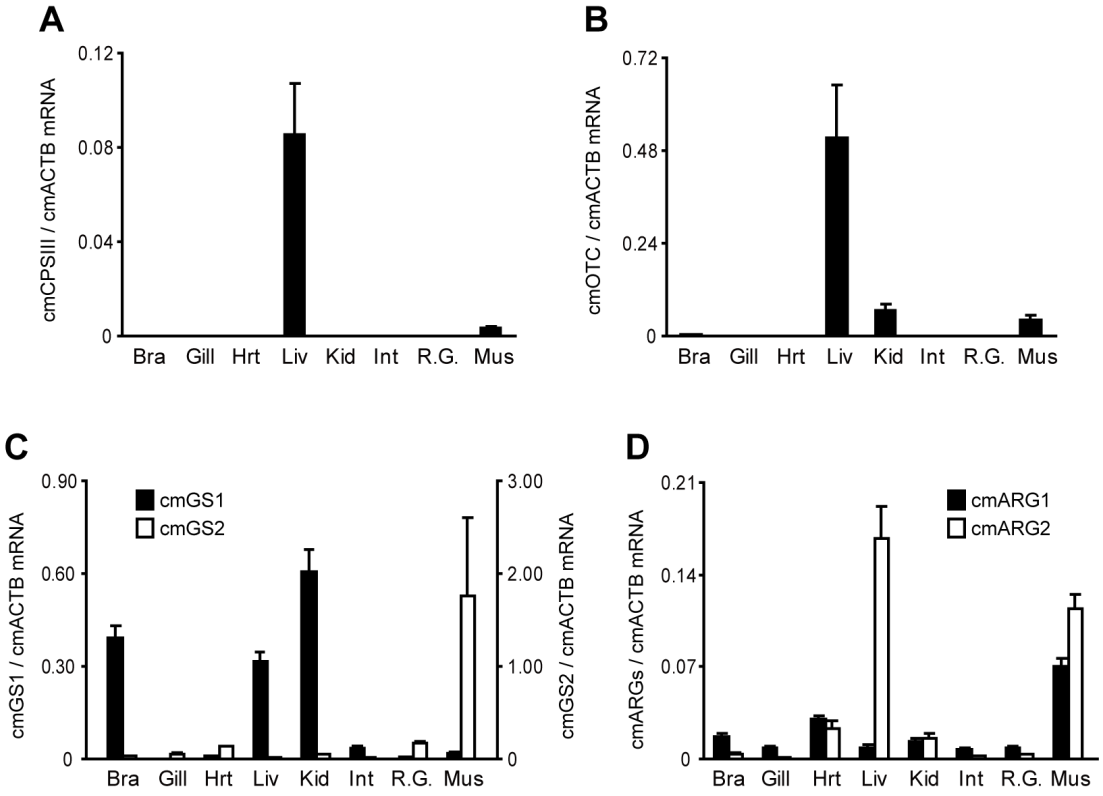

Figure 5. Tissue distribution of mRNA encoding (A) cmCPSIII, (B) cmOTC, (C) cmGS1 and cmGS2, and (D) cmARG1 and cmARG2 from four adult fish (two male and two female). Data are presented as means ± SEM. Values of each mRNA were normalized by the value of cmACTB mRNA. Bra, Brain; Hrt, Heart; Liv, Liver; Kid, Kidney; Int, Intestine; R.G., Rectal gland; Mus, Muscle. Note that the vertical axis for cmGS2 mRNA is longer than that for cmGS1 mRNA in (C).

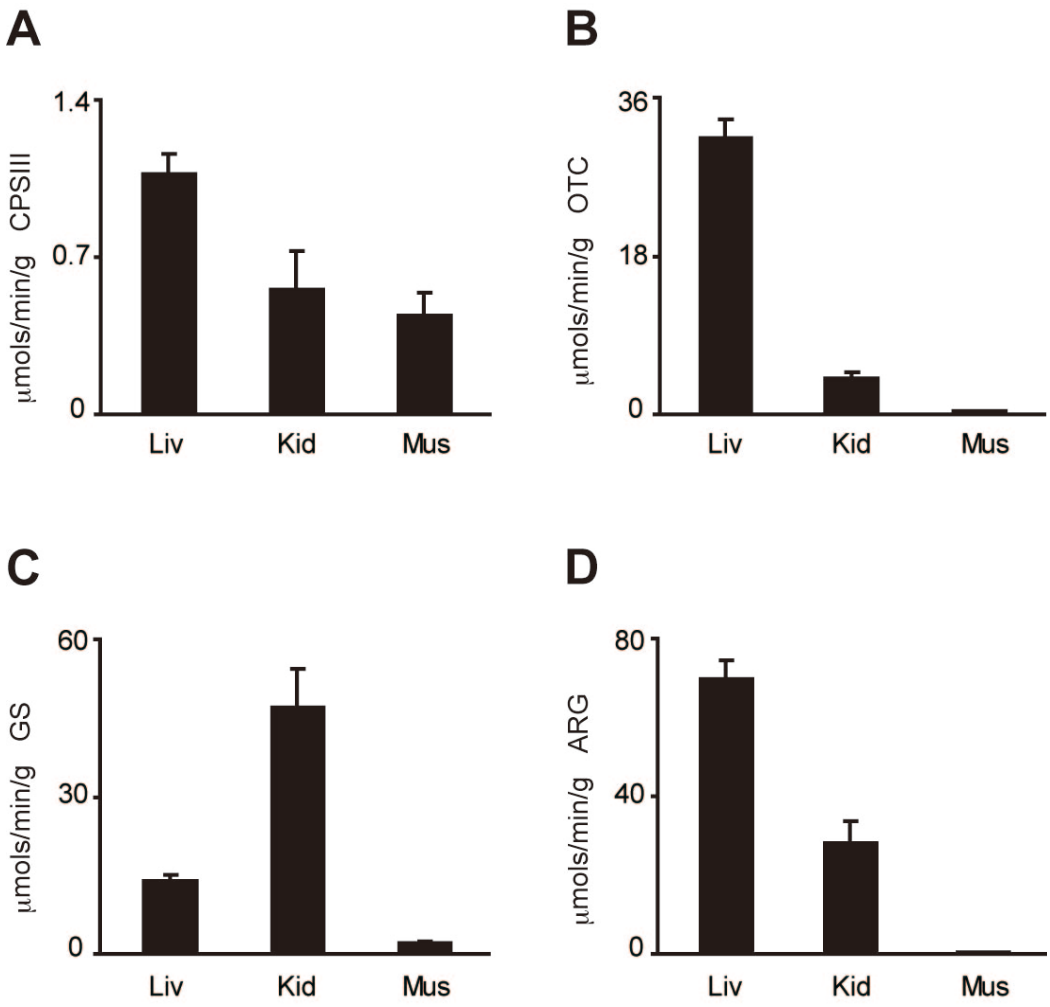

Figure 6. Enzyme activity of (A) CPSIII, (B) OTC, (C) GS, and (D) ARG in liver, kidney and muscle of eight adult fish. Data are presented as means ± SEM. Units are represented as mmol g-glutamyl-hydroxamate formed min^{-1} g^{-1} (GS), mmol citrulline formed min^{-1} g^{-1} (CPSIII and OTC) and mmol urea formed min^{-1} g^{-1} (ARG). Liv, Liver; Kid, Kidney; Mus, Muscle.

Table 2. Changes in the mRNA expression of OUC enzymes and GS in the liver and muscle of elephant fish after transfer to concentrated (120%) and diluted (80%) seawater (SW)

Liver

	Control (100% SW)	120% SW	80% SW
cmCPSIII	23.94 ± 3.64	22.05 ± 3.77	8.15 ± 1.61
cmOTC	89.56 ± 13.66	54.81 ± 15.27	19.31 ± 3.30*
cmGS1	72.74 ± 12.34	53.43 ± 12.44	20.65 ± 2.52
cmGS2	1.57 ± 0.60	3.30 ± 1.08	3.28 ± 1.45
cmARG2	60.09 ± 11.35	74.20 ± 8.99	25.15 ± 1.16

Muscle

cmCPSIII	0.34 ± 0.05	0.36 ± 0.67	0.30 ± 0.14
cmOTC	3.27 ± 1.07	2.59 ± 0.64	1.40 ± 0.45
cmGS1	2.33 ± 0.35	1.93 ± 0.63	0.94 ± 0.11
cmGS2	126.96 ± 49.23	409.07 ± 87.95	228.00 ± 50.66
cmARG2	14.75 ± 2.82	43.45 ± 9.12*	9.66 ± 2.45

Note. Values were normalized by cmACTB, and presented as (means ± SEM) × 100. N = 6 (Control); 6 (120% SW); 4 (80% SW). *, significantly different from control SW group at $P < 0.05$.

Table 3. Plasma parameters of pre-hatching and post-hatching fish

Condition	*N*	Osmolality (mOsm kg^{-1})	Cl^- ($mEq\ l^{-1}$)	Urea ($mmol\ l^{-1}$)	Urea/Osm (%)
pre-hatching	7	1053.9 ± 1.1	251.1 ± 2.9	473.3 ± 8.0	44.9 ± 0.7
post-hatching	6	1054.7 ± 3.9	267.2 ± 8.3	462.8 ± 17.6	43.8 ± 1.5
adult fish[1]	6	1057.3 ± 3.6	285.5 ± 2.0	472.5 ± 16.3	44.7 ± 1.5

Note. Values are means ± SEM. [1]Data from Hyodo et al., 2007.

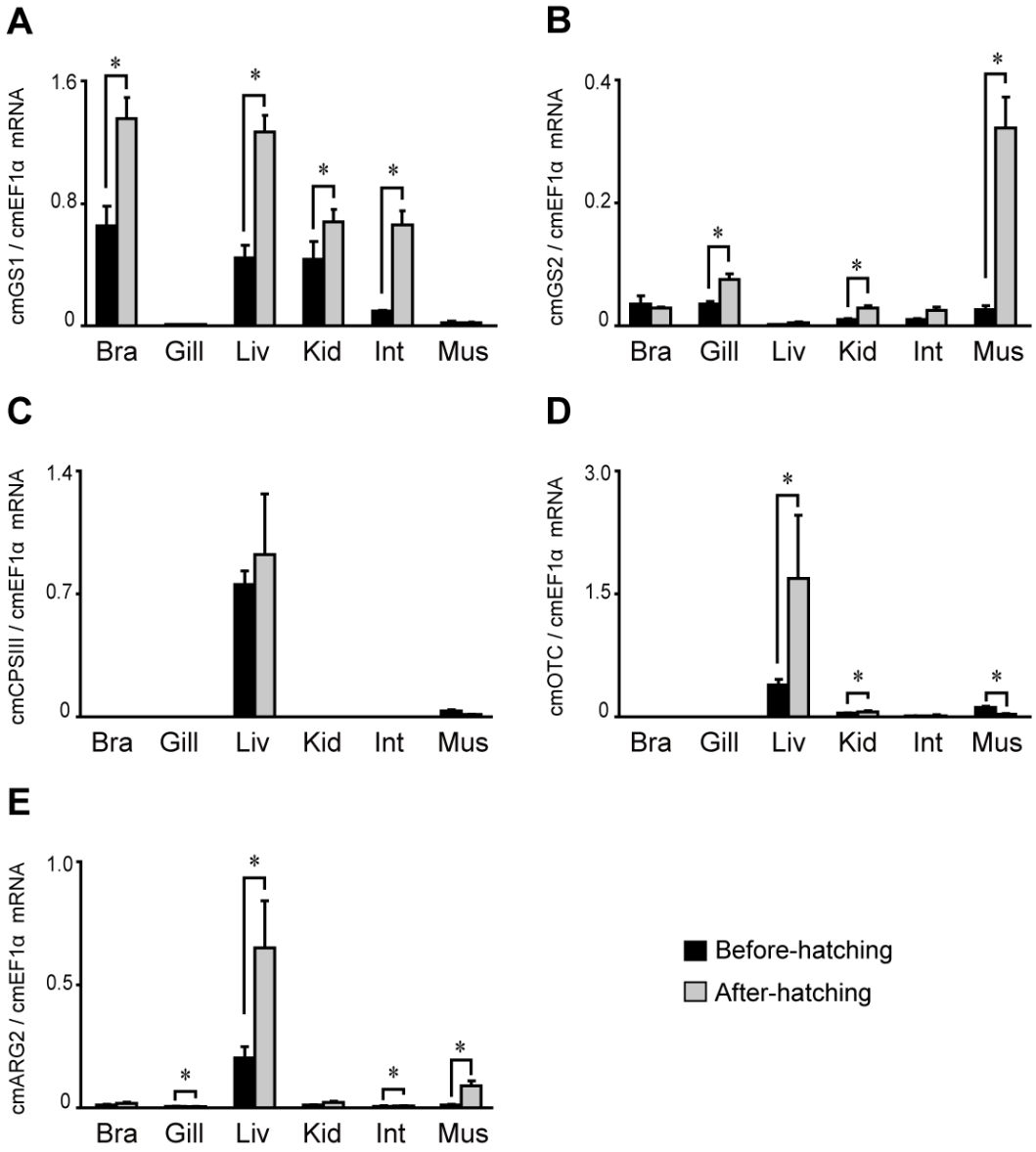

Figure 7. Changes in the mRNA levels of (A) cmGS1, (B) cmGS2, (C) cmCPSIII, (D) cmOTC, and (E) cmARG2, between before and after hatching. Six tissues from three embryos and four hatched fish, were examined. Data were normalized by cmEF1a mRNA levels. Statistically significant differences are shown with asterisks. *$P < 0.05$. Bra, Brain; Liv, Liver; Kid, Kidney; Int, Intestine; Mus, Muscle.

Chapter 2-1

Urea-based osmoregulation in the developing embryos of two oviparous cartilaginous fishes: Contribution of the extra-embryonic yolk sac during the early developmental period in holocephalan elephant fish (*Callorhinchus milii*)

ABSTRACT

Marine cartilaginous fish retain a high concentration of urea to maintain the plasma slightly hyperosmotic to the surrounding seawater. In adult fish, urea is produced by hepatic and extra-hepatic ornithine urea cycles (OUCs). However, little is known about the urea retention mechanism in developing cartilaginous fish embryos. In order to address the question as to the mechanism of urea-based osmoregulation in developing embryos, in this chapter, I examined the gene expression profiles of OUC enzymes in oviparous holocephalan elephant fish (*Callorhinchus milii*) embryos. I discovered that the yolk sac membrane (YSM) makes an important contribution to the ureosmotic strategy of the early embryonic period. The expression of OUC enzyme genes was detectable in the embryonic body from at least stage 28, and increased markedly during development to hatching, which is most probably due to growth of the liver. During the early developmental period, however, the expression of OUC enzyme genes was not prominent in the embryonic body. Meanwhile, I found that the mRNA expression of OUC enzymes was detected in extra-embryonic YSM; the mRNA expression of cmCPSIII in the YSM was much higher than that in the embryonic body during stages 28-31. Significant levels of enzyme activity and the existence of mitochondrial-type cmGS1 transcripts in YSM supported the mRNA findings. I also found that the cmCPSIII transcript is localized in the vascularized inner layer of the YSM. Taken together, these findings demonstrate for the first time that the YSM is involved in urea-based osmoregulation during the early to mid phase of development in oviparous cartilaginous fish.

INTRODUCTION

Marine cartilaginous fish (sharks, skates, rays and chimaeras) are known to retain a high concentration of urea (between 350 and 450 mM) in their extracellular and intracellular fluids for adaptation to the marine environment, and are hence referred to as ureosmotic animals (Smith, 1936; Yancey and Somero, 1980). Urea is produced mainly through the ornithine urea cycle (OUC), comprised of the following five enzymes: rate-limiting mitochondrial carbamoyl phosphate synthetase III (CPSIII), ornithine transcarbamylase (OTC), argininosuccinate synthetase (ASS), argininosuccinate lyase (ASL), and arginase (ARG). Unlike mammalian OUC, mitochondrial glutamine synthetase (GS) is also an important accessory enzyme for the piscine OUC, since CPSIII requires glutamine as its nitrogen-donating substrate instead of ammonia (Anderson, 1980). A number of studies have shown that the liver is the predominant organ for urea production in cartilaginous fish, as it is in other vertebrates (Fänge and Fugelli, 1962; Anderson et al., 2005). On the other hand, recent findings have demonstrated that several extra-hepatic organs, such as muscle, also have a functional OUC and contribute to systemic urea production (Steele et al., 2005; Kajimura et al., 2006; Takagi et al., 2012; Chapter 1).

Meanwhile, little is known about osmoregulation in developing embryos in which the adult organs are not fully developed or are not formed. Cartilaginous fish have a number of breeding strategies from oviparity to viviparity, and their prenatal and egg incubation periods are notably long (Compagno, 1990). Placental and aplacental viviparous species rear their embryos in uteri filled with uterine fluid, whose ionic composition and urea concentration are nearly identical to maternal plasma during early-term pregnancy, and thus requirements for ionoregulation and osmoregulation by the embryos are considered to be minimal during early development (Thorson and Gerst, 1972; Kormanik, 1993). Meanwhile, eggs of oviparous species are laid within three or

four days after fertilization, with the embryos being enclosed in a tough and fibrous egg capsule in SW for the developmental period (Ballard et al., 1993). Although the egg capsule is important for protection from predation, it appears that it does not isolate the intracapsular ionic environment from the external SW (Hornsey, 1978). In addition, at the mid-point of development, one or both anterior sides of the capsule open, and thereafter the capsule fluid is identical to SW for the remaining developmental period (Hamlett and Koob, 1999). This early opening of the egg capsule is commonly seen in oviparous cartilaginous fishes and is called "pre-hatching" (Ballard et al., 1993) or "eclosion" (Hamlett and Koob, 1999). Taken together, embryos in oviparous species have to adapt to the surrounding high salinity intracapsular fluid during the whole developmental period.

In the present study, I used elephant fish (*Callorhinchus milii*) for a developmental study of urea-based osmoregulation in a marine oviparous cartilaginous fish. This species has attracted attention as a model for genome studies of cartilaginous fish (Venkatesh et al., 2005; Venkatesh et al., 2014). In chapter 1, I characterized hepatic and extra-hepatic urea production in adult elephant fish (Takagi et al., 2012). In this chapter, I found that, in addition to the liver of the embryo, the extra-embryonic yolk sac membrane (YSM) contributes to urea production during the early developmental period.

MATERIALS AND METHODS

Embryos

In March 2011, adult elephant fish, *C. milii*, were collected in Western Port Bay, Victoria, Australia, using recreational fishing equipments consisting of a breaking strain line and a hook. Female fish were transported to Primary Industries Research Victoria, Queenscliff in a 1,000 L fish transporter. Fish were kept in a 10,000 L round

tank with running seawater (SW) under a natural photoperiod for approximately two months. During that period, newly laid eggs were gathered from each individual (121 eggs in total), and maintained in a 1,000 L tank with running SW. Embryos in the egg case were sampled in two different seasons, at the beginning of July (developmental stages 28-34) and at the end of September (36 and hatched fish). Mean weights and days post egg-laying (dpe) of embryos at each developmental stage were as follows: 0.12 ± 0.02 g (stage 28, 46-51 dpe); 0.26 ± 0.01 g (stage 29, 58 dpe); 0.38 ± 0.04 g (stage 30, 63-65 dpe); 0.59 ± 0.03 g (stage 31, 67-71 dpe); 1.23 ± 0.11 g (stage 32, 75-82 dpe); 2.01 ± 0.10 g (stage 33, 88-95 dpe); 2.89 ± 0.13 g (stage 34, 94-100 dpe); 13.88 ± 0.84 g (stage 36, 158-178 dpe); 16.83 ± 0.37 g (hatched fish, 182-186 dpe). The developmental stages of elephant fish embryos were identified using an established staging scheme (Didier et al., 1998). In the present study, these developmental stages were roughly divided into three periods: (1) "the early period" in which the external gill filament was extended (stages 28-31); (2) "the middle period" in which the external gill filament was regressed (stages 32-34); and (3) "the late period" in which the external yolk sac was absorbed (from stage 35 to hatching). I confirmed that the pre-hatching (eclosion) occurs at developmental stage 30 in elephant fish. 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 Deakin University.

Sampling of the tissues, body fluid of embryo, and egg capsule fluid

Embryos and larvae were anesthetized in 0.1% (w/v) ethyl 3-aminobenzoate methanesulfonate (Sigma-Aldrich, St. Louis, MO, USA). The tail of the embryo was cut with a razor blade, and blood samples (minimum 4 μl) were obtained from the caudal vasculature with a heparin-coated hematocrit capillary (TERUMO, Tokyo, Japan). Blood samples were centrifuged at 2,250 *g* for 10 min to obtain plasma. Egg

capsule fluid was collected with a syringe. The capsule fluid and blood plasma were stored at -20 ºC until further analysis. Osmolality and sodium concentration were measured with a vapor pressure osmometer (Wescor 5520, Logan, UT, USA) and an atomic-absorption spectrophotometer (Hitachi 180-50, Tokyo, Japan), respectively. Chloride concentration was examined with a digital chloridometer (C-50AP, Jokoh, USA) or by ion chromatography (AV10, Shimadzu, Kyoto, Japan). Urea concentration was measured using a Wako Urea NB test (Wako Pure Chemical Industries, Japan). Embryos were separated from the yolk sac and dissected according to stage. Before stage 32, I could not dissect each organ due to their small size. Therefore, embryonic bodies were roughly separated into two (stage 28: head/body, and tail) or three (stages 30, 31 and 32: head, body, and tail), and after stage 33, the head, gill, liver, gut, muscle and yolk sac membrane (YSM) were dissected out. After stage 36 the brain was dissected. All tissues were quickly frozen in liquid nitrogen, and then stored at -80 ºC. Any remaining tissues following dissection of the above were also frozen for assessing the total abundance of target gene transcripts in the whole body.

Complementary DNA synthesis and RT-PCR

Two micrograms of total RNA were extracted from frozen tissues by guanidium thiocyanate-phenol-chloroform method using ISOGEN (Nippon Gene, Toyama, Japan). After DNase treatment with TURBO DNA-free kit (Life Technologies), first-strand cDNA was synthesized by using a High Capacity cDNA Reverse Transcription Kit (Life Technologies). To examine tissue distribution of the GS mRNAs, RT-PCR was carried out with KAPA Taq Extra DNA polymerase (Kapa Biosystems, Boston, MA, USA) and the specific primer sets for cmGS1 and cmGS2 (Takagi et al., 2012). The primer set for cmGS1 is able to amplify both the long-form and short-form GS mRNAs. Cycle numbers for amplification were 32. Amplified

PCR products were electrophoresed on 1.2% agarose gel, and visualized by ethidium bromide fluorescence. The amplicon size (bp) was determined by a GeneRuler DNA Ladder Mix (Thermo Fisher Scientific, Waltham, MA, USA)

Real-time quantitative PCR assay

Gene expression patterns of OUC enzymes during development were quantified by a real-time quantitative PCR (qPCR) method using a 7900HT Sequence Detection System (Life Technologies, Carlsbad, CA, USA). PCR reactions were performed with KAPA SYBR Fast qPCR kit (Kapa Biosystems) and primer sets of cmCPSIII (GenBank accession no. AB603761), cmOTC (AB622984), cmGS1 (AB622985), cmGS2 (AB622986), cmARG2 (AB622987), which I designed previously (Chapter 1, Takagi et al., 2012). In the present study, I designed a new primer set that was specific for the long-form transcript of cmGS1. Two μL of complementary DNA templates were added to 8 μL of reaction mixture, and measurement was performed in duplicate. To generate a standard curve, plasmids containing partial cDNA fragments of target genes with known concentration were serially diluted and used as the standard templates. Total copy numbers of mRNA (nmol/tissue) in whole embryos and YSM were then calculated for absolute quantification. The amount of short-form transcript of cmGS1 was calculated by subtracting the long-form transcript from the total cmGS1 transcript. Elephant fish elongation factor 1α (cmEF1α, AB622989) was used as an endogenous expression control to calculate relative expression values. The efficiencies for each reaction were 96.5%, 96.0%, 96.4%, 93.6%, 99.5%, 93.2%, 98.6% for cmCPSIII, cmOTC, cmGS1, cmGS1 long-form, cmGS2, cmARG2, cmEF1α, respectively.

In situ hybridization

Whole yolk sac was fixed in modified Bouin's fixative without glacial acetic acid, at 4 ºC for 24h. The YSM was dissected from fixed yolk sac, washed three times with 70% ethanol in order to remove as much yolk as possible, and then embedded in Paraplast (Leica Microsystems, Wetzlar, Germany). Cross sections cut at 8 μm were mounted onto MAS-GP-coated glass slides (Matsunami, Osaka, Japan). For morphological observation, sections were stained with hematoxylin and eosin.

A partial cmCPSIII fragment (1110 bp) was amplified with a gene-specific primer set as follows, GGTTACCCCTGTTTACTGAGG as a sense primer and CCGATAATGATACAGACTGGT as an anti-sense primer, and subcloned into pGEM-T easy (Promega, Madison, WI, USA). Digoxigenin (DIG)-labeled anti-sense cRNA probe was subsequently synthesized by *in vitro* transcription with DIG RNA Labeling Kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. To identify the mRNA localization of CPSIII, *in situ* hybridization was conducted with cRNA probes by using a previously described protocol (Takabe et al., 2012). For a negative control, a DIG-labeled sense cRNA probe was used.

Enzyme activity analysis

Enzyme activity assays were performed on YSM at stages 31 and 34, and on the embryonic liver at stage 36, as described in chapter 1.

Statistical analysis

Data are represented as means ± SEM throughout the study. Unpaired *t* tests were conducted for comparison between mRNA abundance of embryo and YSM, and between enzyme activities of YSM at stages 31 and 34. Steel's non-parametric

rank sum test was conducted for comparison of liver weight and of relative mRNA level in each tissue between the developmental stages. Tukey's test was used for comparison of GS1Long mRNA levels amongst the YSM and several embryonic tissues. *P* values less than 0.05 were considered statistically significant.

RESULTS

Composition of the embryonic body fluid and egg capsule fluid during development

In the present study, I broadly defined development of elephant fish embryos into three periods as described in the Materials and Methods section: (1) "the early period" (stages 28-31) (Fig. 8B, C); (2) "the middle period" (stages 32-34) (Fig. 8D, E); and (3) "the late period" (from stage 35 to hatching) (Fig. 8F). An anterior part of the egg capsule, initially plugged with a dense egg jelly, was opened (pre-hatching) at stage 30 in elephant fish. Osmolality, sodium and chloride levels of egg capsule fluid were similar to the surrounding SW, and the capsule fluid did not contain detectable levels of urea in developmental stages 28-32. No significant difference was observed in osmolality and chloride ion levels between developmental stages even before and after the pre-hatching event, indicating a high permeability of the egg capsule wall to ions and water (Table 4). Body fluid samples obtained from embryos later than stage 31 showed similar concentrations of urea, Na^+ and Cl^- to those of adult fish plasma (Table 5). Before stage 31, I could not obtain enough body fluid for measurements due to the small size; the urea concentration was measurable only from the one fish and was found to be similar to the adult level (Table 5).

Gene expression profile of OUC enzymes in embryos during development

Since the developing embryos contained high levels of urea, the gene expression of the OUC enzymes was examined by quantitative real-time qPCR in order to determine putative urea production sites, using previously established protocols (Chapter 1, Takagi et al., 2012). To this end, the total amount of target gene transcripts was determined for whole embryos (without yolk sac), and calculated as nmol transcript per fish. At stage 28, which was the first stage I sampled, the

expression of all transcripts encoding the OUC enzymes (CPSIII, OTC, GSs and ARG2) was already detectable, and the expression consistently increased throughout development toward the hatching period (Fig. 9).

For embryos of stages 33, 34, 36 and hatched fish, the mRNA levels of the OUC enzymes were examined in the head (brain for stage 36 and hatched fish), gill, liver and muscle (Fig. 10A-E). In this experiment, the mRNA level of the OUC enzymes was normalized against the mRNA levels of elongation factor 1 alpha (EF1α). A high mRNA expression level of the rate-limiting CPSIII was observed only in the liver, while a low level of mRNA expression was detected in the muscle (Fig. 10A). No significant change was observed in the hepatic CPSIII mRNA levels during development. Similar results were observed for both OTC and ARG2 mRNAs, except that the mRNA expression levels were increased in stage 36 and/or hatched fish; the changes were statistically significant for OTC mRNA in muscle and ARG2 mRNA in the liver and muscle (Fig. 10B, E). Meanwhile, the tissue distribution of GS1 and GS2 transcripts showed different patterns. GS1 mRNA expression was observed in the liver, gut and brain, while GS2 mRNA expression was observed predominately in the muscle (Fig. 10C, D). The expression of GS1 and GS2 mRNAs in those tissues was significantly elevated in stage 36 and/or hatched fish except for the GS1 mRNA in the gut. These distribution patterns of OUC enzyme mRNAs in embryonic tissues corresponded well with those in adult elephant fish (Chapter 1, Takagi et al., 2012). All mRNAs encoding OUC enzymes (GS, CPSIII, OTC and ARG) were highly expressed in the liver, implying that the liver is also a major organ for urea production in the embryonic body.

Although the CPSIII mRNA level in the liver was not changed from stage 33 to hatched fish (Fig. 10A), the liver size increased markedly (Fig. 10F), which, at least in part, caused the elevation in the total abundance of CPSIII and other OUC enzymes

mRNAs per fish (Fig. 9).

Gene expression profile of OUC enzymes in yolk sac membrane (YSM)

Although the embryonic expression of OUC enzyme mRNAs were detected from stage 28, their expression levels during the early developmental period were considerably lower than those in the later developmental periods. Since the embryos of early and middle developmental periods are attached to a large yolk sac (Fig. 8B-E), I examined gene expression in the yolk sac membrane (YSM) and found expression of all OUC enzyme mRNAs (Fig. 9). In particular, the expression of the rate-limiting CPSIII mRNA in the YSM was 3.5- to 18-fold higher than that in the embryonic body between stages 28 and 31 (Fig. 9A). The CPSIII mRNA levels in the YSM increased up to stage 31. However, differently from the embryonic body, the CPSIII mRNA levels peaked at stage 31 and then decreased after stage 32. Similar patterns in expression levels in the YSM and embryonic body were also seen for other enzyme mRNAs, although the levels in the YSM were equal or lower than those in the embryonic body even during the early developmental period (Fig. 9B-E).

Figure 11 shows mRNA levels of OUC enzymes in the YSM, which were normalized with the expression levels of EF1α. Consistent with the results of total mRNA abundance (Fig. 9A), the CPSIII mRNA level peaked at stage 30, and then subsequently decreased (Fig. 11A). The expression levels in the early developmental period (stages 29-31) were as high as the levels in the embryonic liver of stage 33. The mRNA levels of OTC and GS1 also tended to decrease in the YSM after stage 30, but the levels in the YSM were less than half of those in the embryonic liver (Figs 11B and C). Similarly, the expression levels of ARG2 were 10 times lower than the level in the liver (Fig. 11E).

Alternative splicing of GS genes in the YSM and embryonic tissues

It is known in cartilaginous fish that two mRNAs with different sizes are transcribed from the GS1 gene by alternative splicing and that those transcripts show distinct subcellular localization (mitochondrial and cytoplasmic; Matthews et al., 2005; Takagi et al., 2012). In adult elephant fish, the long transcript with a putative mitochondrial targeting signal (MTS) is transcribed in the liver, while the short transcript without a MTS was found in the brain and other organs (Chapter 1). Therefore, I examined the tissue distribution of two cmGS1 transcripts with different sizes (GS1Long and GS1Short), together with a cmGS2 transcript in the head, body, tail, and liver (stage 34 only) of the embryonic body and in the YSM at four developmental stages (stage 28, 30, 32 and 34). As in adult fish, two transcripts of cmGS1 with different sizes were found in developing embryos (Fig. 12A). At stage 28, where the embryonic body was separated into head/body and tail parts, a low but observable amount of long transcript was found in the head/body, while expression of the short transcript was predominant in the tail (Fig. 12). For stages 30 and 32, embryos were separated into three parts: head, body and tail. The intense band of long GS1 transcript was detected in the "body" samples, while the short GS1 mRNA was predominantly expressed in the head and tail samples (Fig. 12). These expression patterns of the long-form and the short-form GS1 transcripts were confirmed by quantitative PCR; at stage 32, the expression of GS1Long mRNA in the "body" was the highest amongst the embryonic tissues (Fig. 12B). Since the "body" samples contained the liver, it is most probable that the long transcript in the "body" samples was derived from the liver. At stage 34, I could analyze the liver separately, and found a high expression of GS1Long in the embryonic liver (Fig. 12B), while the short-form of cmGS1 transcript was mainly expressed in the head, tail, and "body", in which the liver was not included (Fig. 12A, C).

The expression of the two alternatively spliced transcripts of cmGS1 was also observed in the YSM. During the early developmental period (stages 28 and 30), in which the expression of CPSIII was high in the YSM (Figs 9A and 11A), the intensity of the band corresponding to the GS1Long mRNA was stronger than that of the GS1Short (Fig. 12A). The results of qPCR analysis revealed that the expression of GS1Long in the extra-embryonic YSM was significantly higher than embryonic tissues (Fig. 12B) and that GS1Long/total GS1 ratio in YSM was around 50% at stage 28 and 30. Meanwhile, in the latter stages (32 and 34), the expression level of the long-form transcript was decreased, resulting in the decrease in GS1Long/total GS1 ratio (Fig. 12A, C).

Activity of OUC enzymes in YSM

In order to determine whether the expressed mRNAs in the YSM are functional, the enzyme activities of CSPIII, OTC, ARG and GS were determined (Table 6). All enzyme activities were detected in the YSM at stages 31 (early period) and 34 (middle period), and the liver of the embryo at stage 36. The value of rate-limiting CPSIII activity in YSM of stage 34 was significantly lower than that of stage 31, and this decrease was consistent with the change in CPSIII mRNA levels (Fig. 11). When the values in the YSM were compared with those in the liver of stage 36 and adult fish (Chapter 1), the values of CPSIII activity were 11 to 28% of the liver samples. Meanwhile, the activities of other enzymes (GS, OTC and ARG) in the YSM were 1.7 to 7% of those in the liver. As a consequence, the ratio between CPSIII and OTC (CPSIII/OTC) was approximately 0.37 in the YSM of stage 31, while it was 0.04 in the liver of stage 36.

Localization of CPSIII mRNA positive cells in YSM

Under the light microscope, YSM was comprised of two cellular layers: an ectodermal outer layer with fibrous connective tissue (FCL), and a vascularized endodermal inner layer (Fig. 13A). Consistent with the previous studies, the endodermal inner layer was closely attached to yolk cytoplasm (Lechenault et al., 1993). Lechenault et al. (1993) showed regional differences in the histological structure of the vascularized YSM wall, suggesting that the YSM is functionally differentiated depending on the area. In the present study, I sampled the proximal area of YSM, which is situated around the yolk stalk and is richly vascularized compared to the distal area. *In situ* hybridization was performed using the YSM of stage 31 when the CPSIII transcript showed the highest expression level (Fig. 9A). CPSIII mRNA positive cells were widely distributed in the vascularized endodermal layer, whereas no signal was observed in the ectodermal layer or the yolk cytoplasm (Fig. 13B); hybridization with the sense probe of CPSIII did not show any positive signals (Fig. 13C). Furthermore, to confirm that the expression of CPSIII mRNA in the YSM is a common phenomenon in oviparous cartilaginous fish, cloudy catshark (*Scyliorhinus torazame*) CPSIII (stCPSIII) cDNA was cloned and the localization of stCPSIII mRNA in the YSM was examined. I used the catshark stage 32 embryo in which the external gill was well-developed (Ballard et al., 1993). As observed in elephant fish, the stCPSIII mRNA signal was only abundantly observed in the vascularized inner layer of YSM (Fig. 13D).

DISCUSSION

In adult cartilaginous fish, past research has revealed the contribution of hepatic and extra-hepatic tissues to urea-based osmoregulation, but little is known about the osmoregulatory mechanisms in the developing embryo. In this chapter, I

confirmed that elephant fish embryos, at least after stage 31, conduct urea-based osmoregulation, and for the first time I revealed that the YSM most probably contributes to urea production during the early developmental period in which the adult organs are not fully developed.

Oviparous cartilaginous fish lay eggs in which the embryo is encapsulated in a collagenous egg capsule. Almost 80 years ago, it was reported that the egg capsule walls of the oviparous lesser spotted dogfish (*Scyliorhinus canicula*) were highly permeable to urea (Needham and Needham, 1930). This property of the egg capsule was further confirmed by studies in which the permeability coefficients of the egg capsule were directly measured in lesser spotted dogfish (Hornsey, 1978) and the big skate (*Raja binoculata*) (Read, 1968b). More recently, it was demonstrated that the osmolality and ionic composition inside the capsule are similar to SW within hours of oviposition (Kormanik, 1992). Conversely, Evans (1981) reported osmotic and ionic gradients between the egg capsule fluid and surrounding SW in oviparous little skate (*Leucoraja erinacea*), suggesting that the egg capsule of this species can be an osmotic and ionic barrier to environmental SW. In the present study, I demonstrated that the egg capsule of the holocephalan elephant fish has a similar property to those of most oviparous sharks; the osmolality and ionic composition of the egg capsule fluid maintained constant levels, which were similar to those of the external environment (SW), throughout development. Therefore, even during early stages prior to opening of the capsule (pre-hatching event), embryos are exposed to a high salinity environment, and this is a common feature of oviparous sharks, skates and chimaeras.

Earlier studies demonstrated considerable amounts of urea in the early-stage embryonic body of several cartilaginous fishes (Needham and Needham, 1930). Read (1968b) further elucidated that the urea concentrations in embryos and yolks are nearly equal in oviparous big skate, and that the urea concentration in the embryos is within

the range of urea levels in body fluid of adult elasmobranchs throughout development. In the present study, I directly measured the urea concentration in the embryonic body fluid (blood plasma); the composition of embryonic body fluid was similar to that in adult fish, suggesting that the elephant fish embryo has an ability to retain urea in their body from the early developmental period. To my knowledge, only a few earlier studies have provided evidence that the developing embryo of oviparous cartilaginous fish produce urea. Read (1968a) demonstrated that big skate embryos have OTC and ARG activities, and that those enzyme activities were increased as development proceeded. More recently, research using little skate showed the presence of CPSIII, OTC, ARG, and GS activities in 4- and 8-month old embryos (Steele et al., 2004). In elephant fish, I detected the embryonic expression of mRNAs encoding a series of OUC enzymes (GSs, CPSIII, OTC and ARG2) from stage 28, in which the external gill starts to extend from the gill slits, and found that the abundance of those transcripts was markedly increased as the hatching stage approached. The change in expression profile is most likely responsible for maintaining the urea level in the embryos despite the increase in body size. Tissue distribution analyses further revealed that the liver is the primary organ for urea production in embryos from at least stage 33 as well as in adult fish (chapter 1). The results imply that the development of the liver contributes to the increase in OUC mRNA abundance in the whole embryo. However, during the early developmental period (stages 28-31), the abundance of OUC enzyme mRNAs in the embryonic body was low compared to that of the later stages.

During the early developmental period, yolk is the largest mass in the egg capsule, and the YSM surrounds the yolk. Therefore, I focused on mRNA expression of OUC enzymes in the YSM. Surprisingly, I found a high abundance of mRNAs encoding OUC enzymes in YSM during the early developmental period (stages 28-31). In particular, the mRNA levels of the rate-limiting CPSIII were much higher in the

YSM compared to the embryonic body. Since the activities of the OUC enzymes can be modulated by post-translational regulation (Nakagawa et al., 2009), the enzyme activities of CPSIII, OTC, ARG and GS were also examined. I confirmed that all of these enzymes are functional in the YSM throughout development; the developmental changes in CPSIII activity corresponded with the changes in CPSIII mRNA expression. These results indicate a considerable contribution of the YSM to urea homeostasis of embryos during early development. A high expression of the long form variant of cmGS1 (GS1Long) with a mitochondrial-targeting signal (MTS) also supports the existence of a functional OUC in the YSM. The long form transcript of GS1, in which an additional exon encoding the MTS is inserted, is expressed in the adult liver, and contributes to piscine OUC by donating glutamine as a substrate for mitochondrial CPSIII in cartilaginous fish (Webb and Brown, 1980; Takagi et al., 2012). The long form transcript of GS1 was also predominantly expressed in the embryonic liver of elephant fish early in development, and most likely contributes to hepatic urea production in embryos. Meanwhile, the cytosolic short form of GS1 appears to contribute to the recycling of neurotransmitters in neural tissue, and for ammonia detoxification in other extra-hepatic tissues (Matthews et al., 2005; Chapter 1). Furthermore, hybridization signals of CPSIII mRNA were found in cells comprising the vascularized endodermal layer of YSM. This localization was further confirmed in the oviparous elasmobranch, cloudy catshark, implying that the expression of CPSIII mRNA in the YSM is a common phenomenon among marine cartilaginous fish. Taken together, my findings indicate that urea is produced in the endodermal layer of YSM, and is then excreted into nearby blood vessels, and subsequently transferred to the embryo in order to maintain a high concentration of urea in the body fluid.

Yolk sacs are found in many vertebrates, and the extra-embryonic YSM has been considered to play a key role in absorption of yolk nutrients (Lambson, 1970; Diez

and Davenport, 1990; Lechenault et al., 1993; Zohn and Sarkar, 2010; Bauer et al., 2013). An intriguing finding on the role of the YSM in teleost fish is that it is involved in osmoregulation during early development (Kaneko et al., 2008). Mitochondria-rich ionocytes, which are responsible for active ionic regulation in adult gill epithelia, can be observed in the YSM during the early development of embryos reared in either SW or FW environments (Ayson et al., 1994; Hiroi et al., 2008), suggesting that these extra-branchial ionocytes are important for body fluid homeostasis of the developing embryos of teleosts. The presence of ionocytes involved in ion and acid-base regulation in the YSM has also been reported in squid *Sepioteuthis lessoniana* and cuttlefish *Sepia officinalis* (Hu et al., 2011). Although the origin of ionocytes and endodermal urea-producing cells appear to be different, the present finding that cartilaginous fish YSM expresses a functional OUC strongly suggests that the extra-embryonic YSM of aquatic animals makes an important contribution to environmental adaptation in general.

One of the interesting observations in the YSM is that the relative mRNA expression and activity of CPSIII to other enzymes were considerably higher in the YSM compared to the liver of adult elephant fish. In general, the mRNA expression and activity of CPSIII is lower than the other OUC enzymes, such as OTC and ARG, and thus CPSIII has been referred to as a rate-limiting enzyme in the piscine OUC (Janssens and Cohen, 1968). In the liver of adult elephant fish, the ratio between CPSIII and OTC activities (CPSIII/OTC) was approximately 0.034 (Takagi et al., 2012; chapter 1), and this value is consistent with the values calculated for other species (Kajimura et al., 2006). In contrast, in the YSM of elephant fish, the CPSIII/OTC activity ratio was approximately 0.37 (stage 31) and 0.17 (stage 34). The high ratio obtained in the YSM most probably reflects the high relative activity of CPSIII in the YSM *in vivo*. Further research is necessary to clarify why such a high activity of

CPSIII to other OUC enzymes is required in the YSM, and whether the high ratio is a common phenomenon in oviparous cartilaginous fishes.

In the present study, I examined embryos from stage 28 because at the earlier stages of development the embryos were too small for analysis. Further research is necessary to investigate how the YSM forms and at what stage the endodermal cells begin to express OUC enzyme transcripts. The contribution of maternal OUC enzyme mRNAs and/or maternal urea in yolk to embryonic osmoregulation during the initial developmental period is also of interest. Read (1968b) showed in big skate that the fertilized egg and entire embryonic system (embryo plus yolk) contain notable amounts of urea during the early developmental period.

In summary, I found that the embryos of holocephalan elephant fish conduct urea-based osmoregulation from the early stages of development, and that the YSM most probably makes an important contribution to urea production, particularly during the early developmental period in which the liver of the embryo is not sufficiently developed. After stage 32, the abundance of OUC enzyme mRNAs was dramatically increased in the liver as hatching approached, while the abundance of OUC enzyme mRNAs in the YSM decreased, which reflects both the decrease in expression level in the YSM and the regression of the yolk sac. These results imply a functional shift of the urea production site during embryonic development from the YSM to the embryonic body (liver) at around stage 32. In teleosts, functional ionocytes are distributed in the YSM during early embryonic stages (Kaneko et al., 2008), and a distributional shift of ionocytes from the YSM to the gills occurs (Hiroi et al., 1998; Katoh et al., 2000). Similar ontogeny-dependent shifts in the site of osmoregulation have also been reported in marine crustaceans (Cieluch et al., 2005) and cephalopods (Hu et al., 2011). Although the mechanisms to regulate body fluid homeostasis are different among aquatic species (cartilaginous fish, teleost fish and cephalopod), it is reasonable to

suggest that the YSM is a critical osmoregulatory organ in aquatic animals during early development.

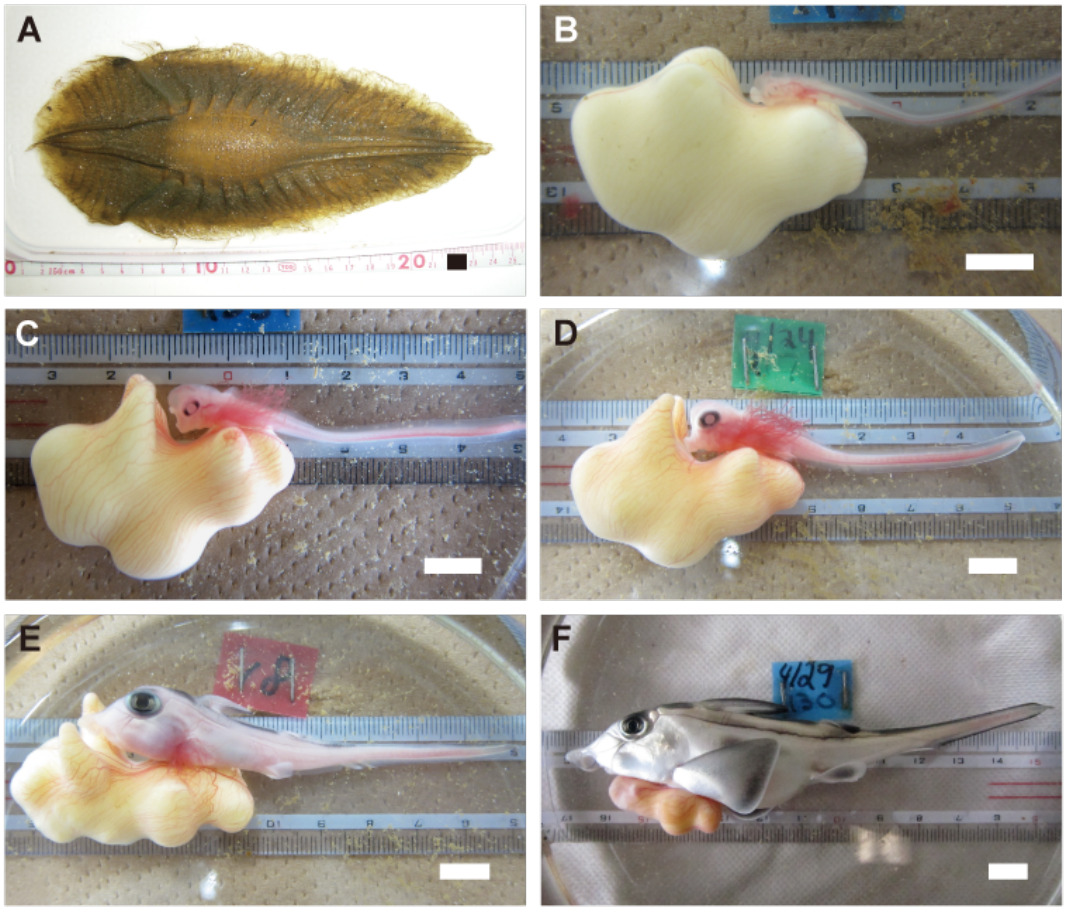

Figure 8. An egg capsule and a series of embryos at different developmental stages of elephant fish. Developmental stages were identified according to Didier et al. (Didier et al., 1998). (A) A tough and fibrous egg capsule, which is a common feature of oviparous cartilaginous fishes. (B) Embryo at stage 28. (C) Embryo at stage 30 showing dark pigmentation around the lens of the eye. (D) Stage 32. Around this stage, external gill filaments reach the maximum length and then begin to regress. (E) Embryo at stage 34 representing regression of the external gill filaments. (F) Embryo at stage 36 with fully formed fins and no external gill filaments. In the later period of stage 36, the external yolk sac is completely absorbed. Scale bar = 1cm.

Table 4. Composition of egg capsule fluid

Egg capsule fluid	*N*	Osmolality (mOsm kg^{-1})	Cl^- (mEq l^{-1})	Urea (mmol l^{-1})
At stage 28	4	1033.0 ± 19.3	532.3 ± 10.2	ND
29	4	1023.5 ± 16.8	534.3 ± 9.1	ND
30 (pre-hatching period)	5	1033.0 ± 11.3	534.6 ± 7.3	ND
31	6	999.3 ± 18.7	527.2 ± 5.1	ND
32	4	994.5 ± 27.4	531.5 ± 9.0	ND
SW		1054.0	574.0	ND

Note. Values are means ± SEM. ND, not detectable.

Table 5. Composition of embryonic body fluid

Body fluid	*N*	Osmolality (mOsm kg^{-1})	Na^+ (mmol l^{-1})	Cl^- (mmol l^{-1})	Urea (mmol l^{-1})
At stage 31	1	-	-	-	449
32	6	-	284.5 ± 7.0	290.8 ± 8.0	488.6 ± 13.1
33	7	-	308.4 ± 5.6	307.2 ± 5.4	498.2 ± 8.1
34	3	-	327.3 ± 1.6	295.5 ± 4.3	480.7 ± 7.4
36	12	1048.9 ± 3.1	298.7 ± 4.2	316.3 ± 11.1	473.3 ± 8.0
Hatched fish	5	1055.2 ± 3.4	337.9 ± 15.6	339.1 ± 17.5	462.8 ± 17.6
Adult fish[1]	6	1057.3 ± 3.6	-	285.5 ± 2.0	472.5 ± 16.3

Note. Values are means ± SEM. [1,] Data from Hyodo et al., 2007. Developmental stage 36 in this chapter corresponds to pre-hatching ("before hatching") fish in chapter 1.

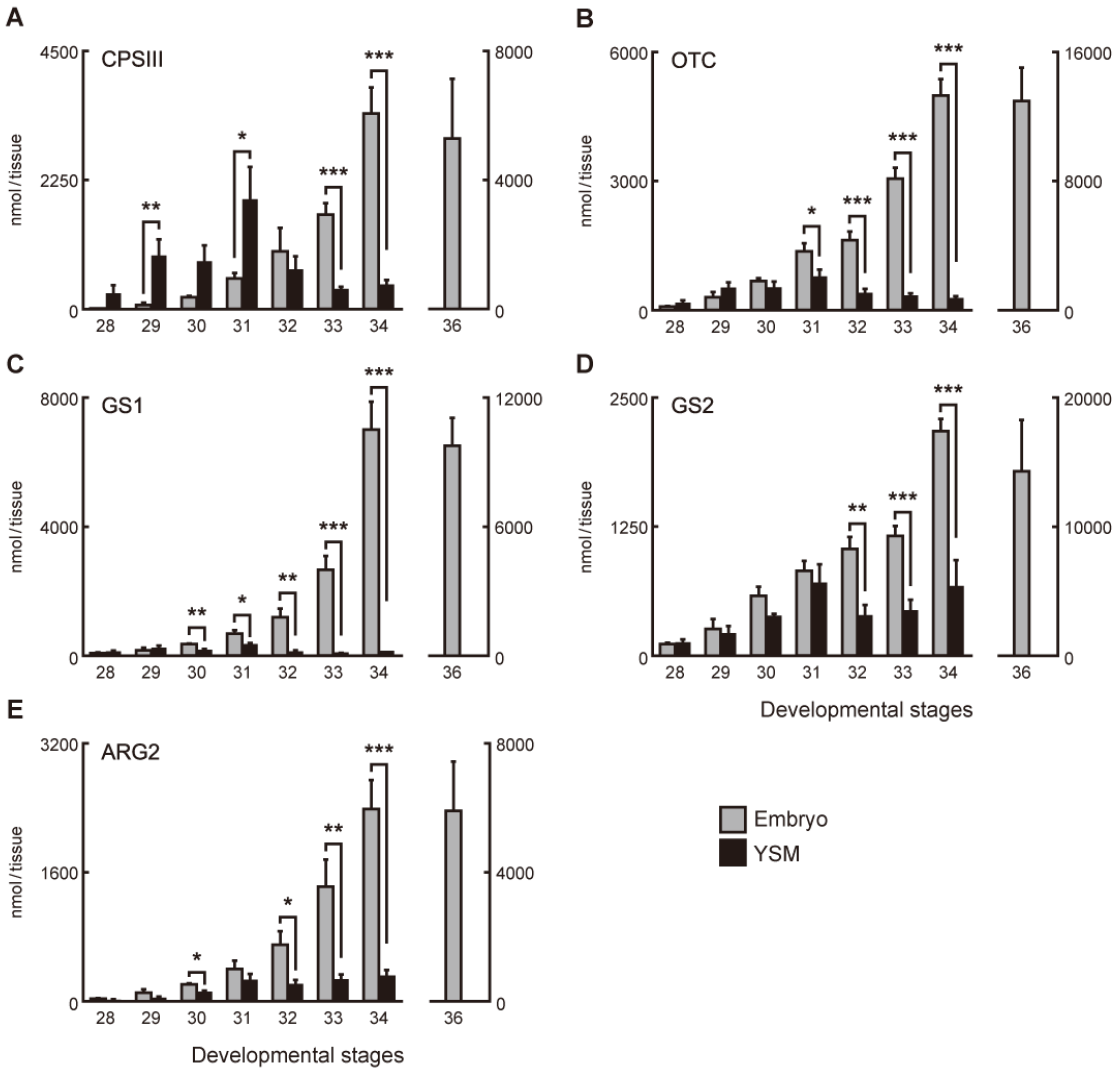

Figure 9. Developmental changes in total mRNA abundance (nmol/fish or YSM) of cmCPSIII (A), cmOTC (B), cmGS1 (C), cmGS2 (D), and cmARG2 (E) in whole embryo and the YSM. Data are presented as means ± SEM. $N = 4$ (developmental stage 28); 3 (stage 29); 3 (stage 30); 6 (from stage 31 to 34). Asterisks indicate significant differences (*$P < 0.05$; **$P < 0.01$, ***$P < 0.001$) in the mRNA abundance between whole embryo and YSM in each stage.

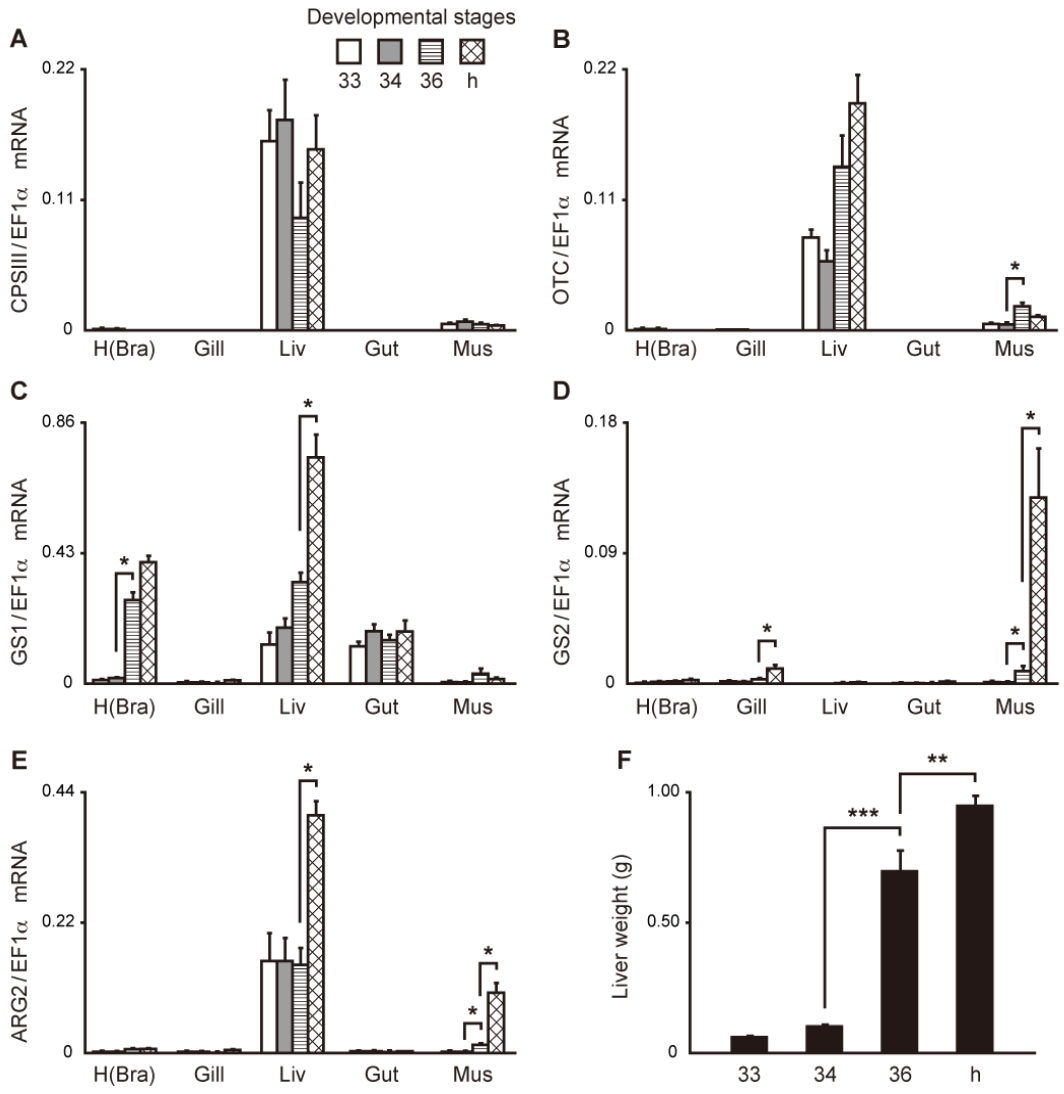

Figure 10. Developmental changes in relative mRNA levels of cmCPSIII (A), cmOTC (B), cmGS1 (C), cmGS2 (D), and cmARG2 (E) in tissues of embryos. Values of each mRNA were normalized against the value of cmEF1α mRNA as an internal control gene. Developmental increase in embryonic liver weight is shown (F). All data are presented as means ± SEM. $N = 6$ for all samples (stage 33 to hatched fish). H (Bra), head (stage 33 and 34) or brain (stage 36 and hatching); Liv, liver; M, muscle; h, hatched. Asterisks indicate significant differences (*$P < 0.05$; **$P < 0.01$, ***$P < 0.001$) in the mRNA levels between adjacent stages.

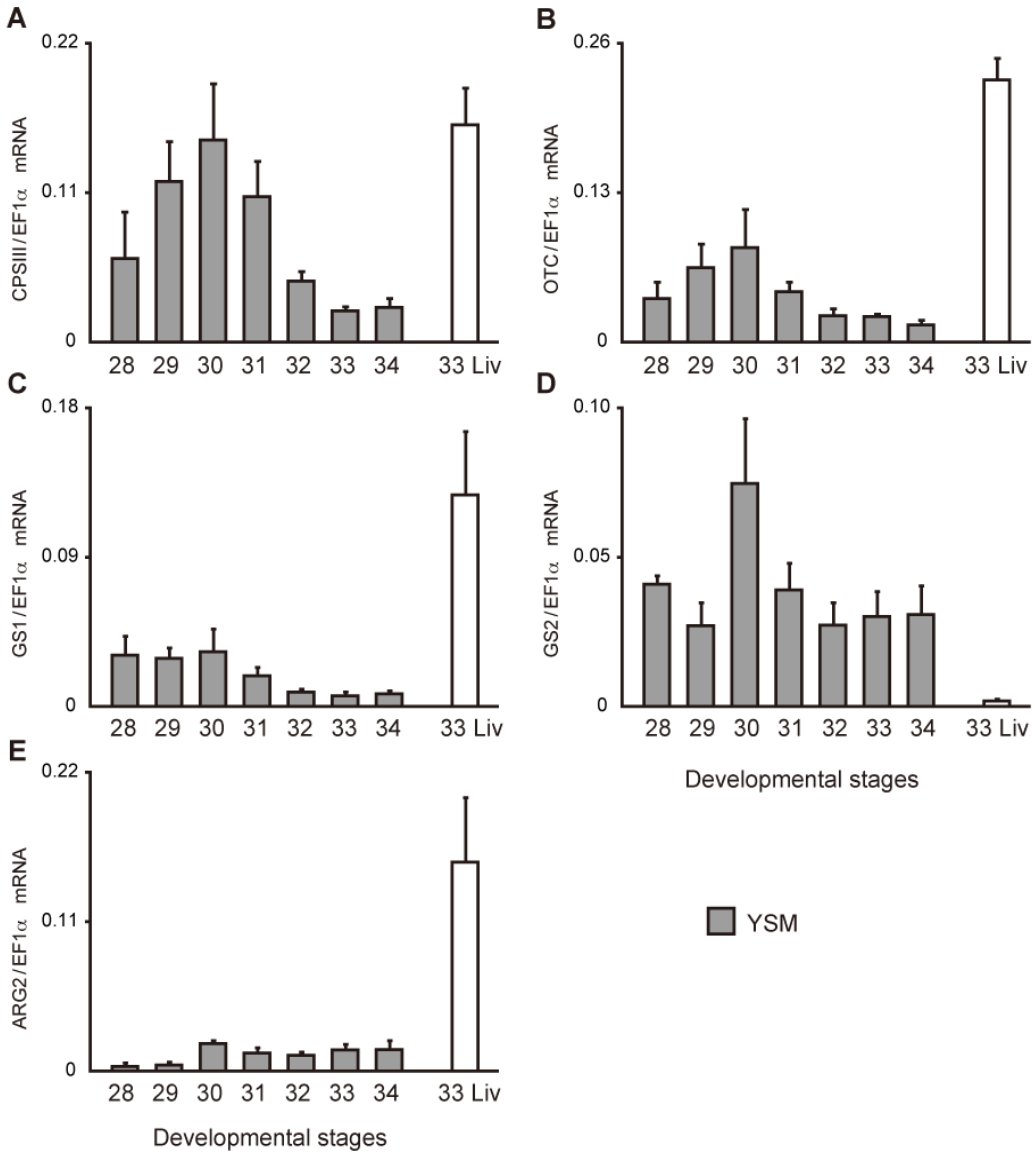

Figure 11. Developmental changes in relative mRNA levels of cmCPSIII (A), cmOTC (B), cmGS1 (C), cmGS2 (D), and cmARG2 (E) in YSM. Each value was normalized by cmEF1α as described in Figure 10. All data are presented as means ± SEM. N = 4 (developmental stage 28); 3 (stage 29); 3 (stage 30); 6 (from stage 31 to 34). For comparison, mRNA levels in the liver of stage 33 were also shown (N = 6). YSM, yolk sac membrane; Liv, liver.

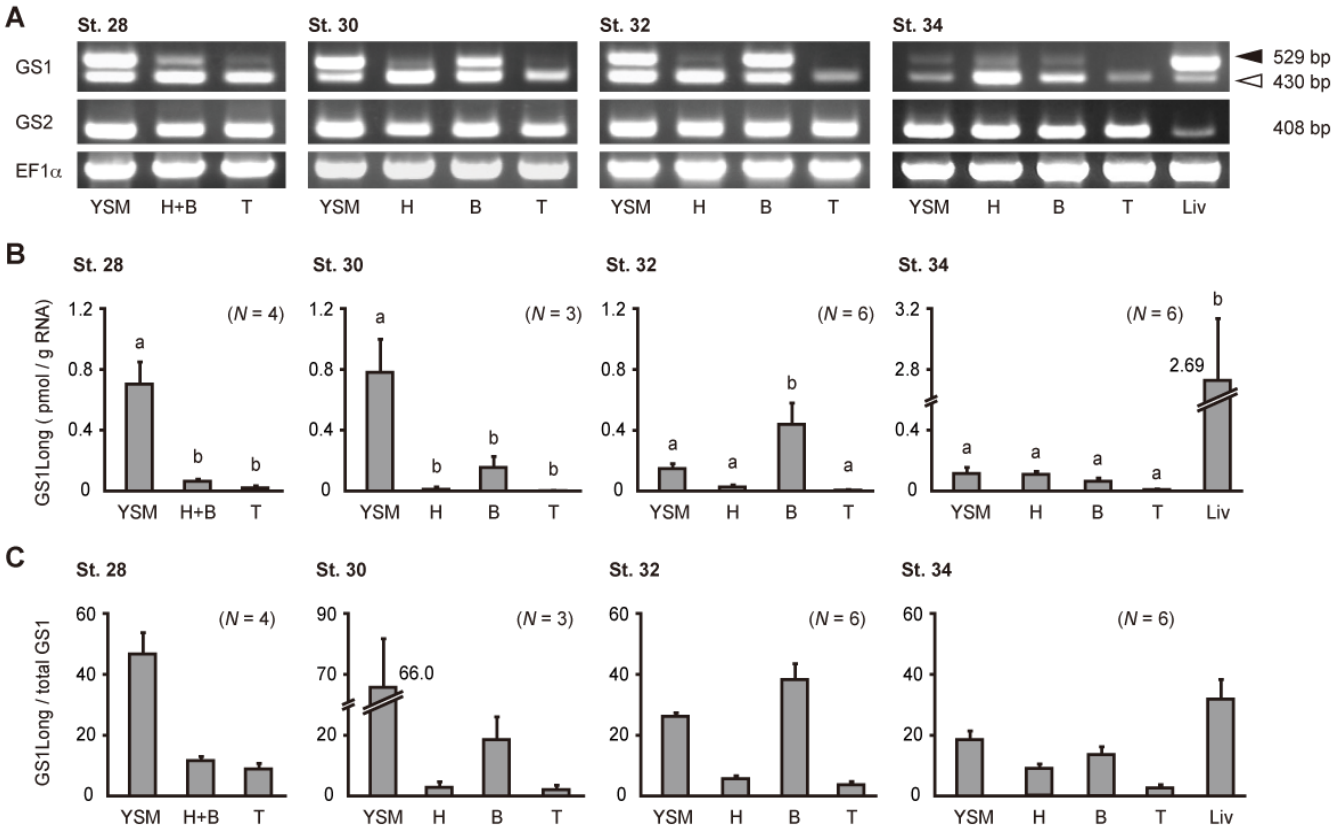

Figure 12. Expression patterns of cmGS transcripts (cmGS1Long, cmGS1Short and cmGS2) and their changes during development. The cmEF1α mRNA was used as a positive control for each cDNA sample. Black and white arrowheads indicate cmGS1Long and cmGS1Short mRNAs, respectively. Amplicon lengths of cmGS1Long, cmGS1Short and cmGS2 were also shown. Note that the "body" sample of embryo at stage 34 does not contain the liver. YSM, yolk sac membrane; H, head; B, body; T, tail; Liv, liver; St., developmental stage.

Table 6. Activities of OUC enzymes

Samples	GS	CPSIII	OTC	ARG
YSM at stage 31	0.43 ± 0.16	0.24 ± 0.04	0.65 ± 0.18	1.17 ± 0.25
YSM at 34	0.34 ± 0.06	0.12 ± 0.04 *	0.73 ± 0.10	1.72 ± 0.19
Liver at 36	15.53 ± 0.92	0.87 ± 0.13	21.88 ± 1.45	23.74 ± 2.26
Adult liver [1]	13.84 ± 1.09	1.07 ± 0.09	31.36 ± 2.12	69.79 ± 4.47

Values were presented as means ± SEM. $N = 5$ (YSM at stage 31); 5 (YSM at 34); 6 (embryonic liver at 36). *, significantly different in CPSIII activities of YSM between stages 31 and 34 at $P < 0.05$. [1]Data of adult fish liver were from chapter 1.

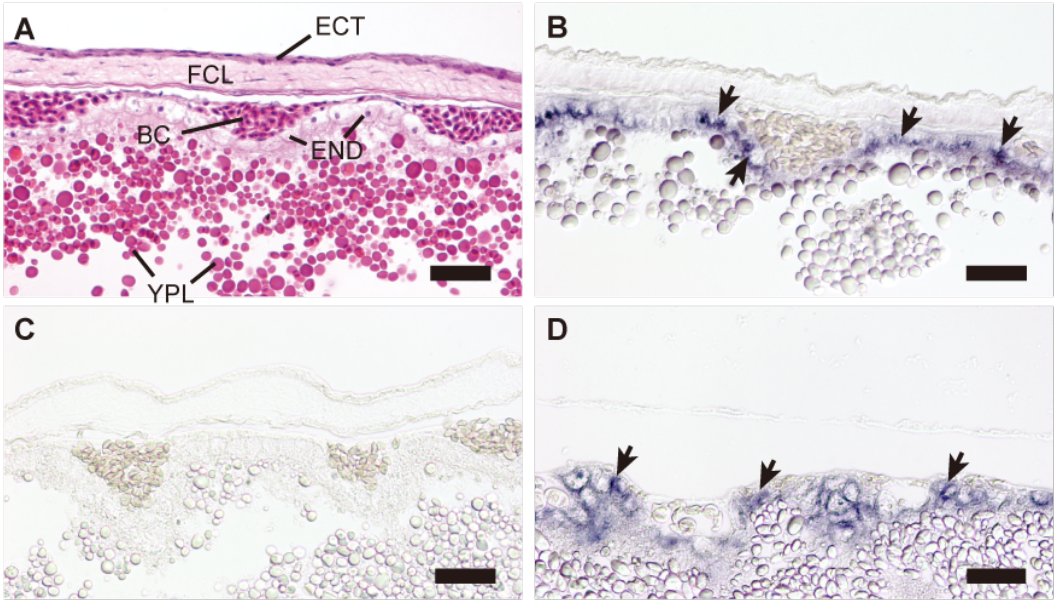

Figure 13. Morphological observations of the YSM shown by HE staining (A) and localization of cmCPSIII mRNA by *in situ* hybridization (B, C). D, Localization of cloudy catshark (*Scyliorhinus torazame*) CPSIII (stCPSIII) mRNA confirmed the expression of CPSIII mRNA in cells of the vascularized endodermal layer of the YSM. Black arrows indicate positive signals of cmCPSIII (B) and stCPSIII (D) transcripts, respectively. ECT, ectoderm; FCL, fibrous connective layer; END, endoderm; BC, blood cell; YPL, yolk platelets. Scale bars, 50 μm.

Chapter 2-2

Urea-based osmoregulation in the developing embryos of two oviparous cartilaginous fishes: Distributional shift of urea production site from the extra-embryonic yolk sac membrane to the embryonic liver during the development of catshark (*Scyliorhinus torazame*)

ABSTRACT

As described in chapter 2-1, I found the important contribution of extra-embryonic yolk sac to urea production during the development of oviparous holocephalan elephant fish. In this chapter, I used cloudy catshark embryos, which belong to another subclass in Chondrichthyes, the Elasmobranchii, and investigated the role of yolk sac membrane (YSM) in urea production during development. The concentration of urea both in yolk and embryonic body were maintained at levels similar to adult plasma concentration throughout the development, confirming that catshark embryos also conduct urea-based osmoregulation. The results of quantitative PCR analyses demonstrated that the mRNA expression of catshark glutamine synthetase, carbamoyl phosphate synthetase III (stCPSIII), ornithine transcarbamylase and arginase was kept at higher levels in YSM than in embryo until stage 32, and that all ornithine urea cycle (OUC) enzymes were predominantly expressed in the mesendodermal layer of YSM. The highest enzyme activities were also found in the mesendodermal layer of YSM. The mRNA abundance of OUC enzymes, in particular the rate-limiting stCPSIII, was rapidly decreased after stage 32, while that in the embryonic body was markedly increased. The important contribution of YSM and embryonic body to urea production in the early-middle and late developmental phases, respectively, was also implied by the results of OUC enzyme activities. Taken together, the results in this chapter support my idea that the YSM of oviparous cartilaginous fishes contributes to embryonic urea homeostasis until the liver becomes fully functional, in order to cope with high-salinity and high-osmolality external environments.

INTRODUCTION

For adaptation to the high-salinity and high-osmolality marine environment, cartilaginous fishes (elasmobranchs and holocephalans) retain a high concentration of urea as an osmolyte to control their plasma slightly hyperosmotic to surrounding seawater (SW). They do not suffer dehydration even in the high-osmolality SW environment, and thus urea is thought to be one of the most important osmolytes in marine cartilaginous fishes (Smith, 1936). A large body of research has demonstrated that, in adult fish, several omoregulatory organs, such as liver, muscle, gill and kidney, function in a coordinated manner for this unique urea-based osmoregulation (Boylan, 1972; Anderson, 2001; Fines et al., 2001). Furthermore, previous studies demonstrated that embryos of both oviparous and viviparous species contain a considerable amount of urea (Hunter and Dauphinee, 1924; Needham and Needham, 1930; Read, 1968b), suggesting that embryos of cartilaginous fishes also conduct urea-based osmoregulation as well as adult fish. However, the urea-retaining mechanisms in embryos in which the adult osmoregulatory organs are not fully developed have been poorly understood.

In chapter 2-1, I demonstrated that embryos of oviparous holocephalan elephant fish conduct urea-based osmoregulation as in adult fish, and found for the first time that extra-embryonic yolk sac membrane (YSM) makes comparable or even greater contribution to urea production, compared to the contribution of the embryonic body, during the early developmental period. Furthermore, I found that the urea-producing site is shifted from the YSM to the embryonic liver at later developmental stages. An upcoming question is whether my findings in elephant fish embryos are a generally observed phenomena among oviparous cartilaginous fishes. To address this question, I focused on an oviparous elasmobranch, the cloudy catshark (*Scyliorhinus torazame*). The use of catshark provides several

advantages; the catshark lays two eggs once every a five to sixteen days (Mellinger, 1983), and the catshark does not have a particular breeding season. In collaboration with an aquarium, I therefore could obtain a large number of fertilized eggs throughout the year. The obtained eggs were able to be cultivated in a tank in the laboratory. Furthermore, in collaboration with Dr. Kuraku of RIKEN CDB, I could use a preliminary genome database of the cloudy catshark. Using catshark embryos, I first measured urea contents in the embryonic body and the yolk of various developmental stages to confirm that catshark embryos use the ureosmotic strategy. Similar to elephant fish, I found a considerable contribution of YSM to urea production, and a distributional shift of urea production site from the YSM to the embryonic liver in catshark.

MATERIALS AND METHODS

Animals

Sexually matured cloudy catshark (*S. torazame*) (Total length 39-45 cm, body weight 280-500g) and laid eggs were transported from Japanese public aquarium (Aqua world Ibaraki Prefectural Oarai Aquarium) to the Atmosphere and Ocean Research Institute, University of Tokyo. They were kept in a 3,000 L rectangular tank (adult fish) or in floating basket in an 1,000 L tank (eggs) at 16 ºC under a constant photoperiod (14 L: 10 D). Adult fish were fed squid once a week. In total, 192 eggs were used in this study. In order to identify developmental stages, a detailed table of developmental stages of a closely related species, *S. canicula*, was used (Ballard et al., 1993). All animal experiments were conducted according to the Guidelines for Care and Use of Animals approved by the committee of the University of Tokyo.

Tissue sampling

All animals used in the present study were anesthetized in 0.1% (w/v) ethyl 3-aminobenzoate methanesulfonate (Sigma-Aldrich, St. Louis, MO, USA). The liver was dissected out from four adult catsharks (male = 2, female = 2). Developing embryos of stages 20-23 (N = 9), 24-26, 33, 34 (N = 5 for each stage), 27 (N = 4), 28 (N = 19), 29 (N = 6), 30, 31, 32 (N = 16 for each stage) and hatched fish (N = 5) were used for various analyses. After opening the egg capsule, intracapsuler fluid was collected with a syringe. From stages 20 to 32, whole embryo, yolk, and YSM were sampled for developmental analyses of mRNA levels; after stage 33, embryonic body was dissected to obtain the brain, liver, gill, muscle, digestive tract (gut) and kidney. For distribution analysis, ectodermal and mesendodermal cell layers of YSM were sampled separately from stage 30 to 31 (N = 12 in total). Whole embryos and YSM at stages 28, 30 and 32 were used for measuring the enzyme activities. Two cell layers of the YSM at stage 32 were individually sampled as described above. Unfertilized eggs (N = 3) were also collected from the oviduct of adult fish. All tissue samples were flash frozen in liquid nitrogen and stored at -80 ºC until use for RNA extraction and measurement of enzyme activities, and at -30 ºC for measurement of urea concentration.

Osmolality and chloride concentration of the intracapsuler fluid were measured with a vapor pressure osmometer (Wescor 5520) and a digital chloridometer (C-50AP), respectively. For measurement of urea concentration in embryo and yolk, samples were mixed with water and homogenized using Micro Smash MS-100 (TOMY SEIKO, JAPAN) as follows: 29 volume (ml) of water to 1 weight (g) of embryo (w/v), and 19 volume (ml) of water to 1 volume (ml) of yolk (v/v). Supernatant was collected by centrifugation at 12,000 rpm for 30 minutes at 4 ºC, and the urea concentration was measured by urease indophenol method using a

Wako Urea NB test (Wako Pure Chemical Industries).

Molecular cloning

Cloning of catshark ornithine urea cycle (OUC) enzyme cDNAs from the frozen liver was performed with the same methods as described in chapter 2-1. In brief, total RNA was extracted from the adult liver with ISOGEN (Nippon Gene), and contaminated genomic DNA was digested by TURBO DNA-free kit (Life technologies). Complementary DNA was synthesized from DNase-treated RNA using a High Capacity cDNA Reverse Transcription Kit (Life technologies). Partial cDNA fragments of the target genes were amplified by PCR, using primer sets shown in Table 9. The resulting PCR amplicons were ligated into pGEM T-easy vector (Promega) and sequenced by a DNA sequencer (ABI PRISM 3130, Life technologies). Five-prime and three-prime regions of catshark (st) glutamine synthetase (stGS) and arginase (stARG) cDNAs were determined by 5'-RACE and 3'-RACE methods using SMARTer RACE cDNA Amplification Kit (Clontech) and gene-specific primer sets (Table 9).

Real-time quantitative PCR assay

Tissue distribution and developmental changes of OUC enzyme mRNAs were analyzed by a real-time qPCR using a 7900HT Sequence Detection System (Life Technologies) as described in chapter 2-1. For calculation of relative expression values, a house keeping gene, β-actin (stACTB) was used as an internal control. The absolute quantification of mRNA abundance was conducted as described in chapter 2-1.

Development of embryonic liver

Embryos of stage 23 (Group 1), 24-26 (Group 2), 27-28 (Group 3), 31 (Group 4), 32 (Group 5), and hatched fish (Group 6) were fixed in Bouin's solution and embedded in Paraplast (Leica). Serial cross sections cut at 8 μm were mounted onto gelatin-coated slides, and stained with hematoxylin and eosin. The liver volume of each sample was calculated by integrating liver area of the cross sections by ImageJ (Abràmoff et al., 2004).

Enzyme activities of OUC enzymes

Activities of GS, carbamoyl phosphate synthetase (CPSIII), ornithine transcarbamylase (OTC), and ARG in catshark embryo and YSM at stages 28, 30, and 32 ($N = 6$) were measured as described in chapter 1. For stage 32, ectodermal and mesendodermal layers of YSM were separately sampled and measured. The minimum detection limits of CPSIII and OTC activities were calculated as 0.023 and 0.052 μmol min^{-1} g^{-1}, respectively.

Statistical analysis

Data are represented as means ± SEM. Data from the enzyme activities and urea concentration were analyzed by Tukey's test. Steel's non-parametric rank sum test was used for analysis of the data of osmolality and chloride concentration, and for comparison of the mRNA expressions between the embryo and the two cell layers of YSM. A Mann-Whitney *U* test was conducted for comparison between relative mRNA levels of developing embryo and YSM. Meanwhile, an unpaired *t* test was conducted for the mRNA abundances between the embryos and YSM. *P* values less than 0.05 were considered statistically significant.

RESULTS

Intracapsular environment and urea concentration of developing embryos

Developmental stages of cloudy catshark (*S. torazame*) were determined by observation of distinct external features, for example, fin folds, pigmentation, length of external gill filaments, and yolk regression according to the table of developmental stages for *S. canicula* (Ballard et al., 1993) (Fig. 14). The wet weights of embryo and yolk, and the length of external gill filaments were shown in Table 7. The external gills reach the maximum length at stage 32, while pigment begins to appear in the skin during the latter half of stage 32. The external yolk is obviously shrinking in size at stage 33.

In catshark, early opening of the egg capsule (pre-hatching) was observed during developmental stage 31. Figure 15 shows chloride ion concentration and osmolality of the intracapsular fluid from stage 20 to 34. The data clearly showed that intracapsular salinity and osmolality were nearly identical to levels of SW, and almost invariable throughout the development, regardless of whether egg capsule was tightly closed or not (Fig. 15).

Urea concentration in the embryonic body and yolk was also measured from stage 28 to 32 (Table 8). The extracts of embryo showed high urea levels ranging from 324.5 to 379.7 mM. The values were nearly constant throughout the development, and were similar to the plasma urea levels of adult fish. Meanwhile, urea levels in yolk ranged from 220.7 to 262.4 mM. After stage 29, absorption of yolk gradually progressed; however, the urea concentration in yolk was nearly constant throughout the development. The urea concentration of undeveloped eggs was also nearly identical to that in the yolk after stage 28. The urea concentration in embryo was higher than that in yolk at any developmental stage.

Molecular cloning of catshark OUC enzyme genes

The partial cDNA sequences of CPSIII, OTC, Argininosuccinate synthetase (ASS), Argininosuccinate lyase (ASL) were determined (Fig. 16). Although two distinct genes encoding GSs (cmGS1 and 2) were found in elephant fish, only transcripts of a single GS gene were found in catshark (stGS). However, three alternatively spliced transcripts of catshark GS gene with different sizes were obtained (Fig. 17A). The cDNAs encoding the entire coding region of stGS were then obtained and their sequences were determined. The deduced amino acid sequence of catshark stGS cDNA exhibited higher similarity to elephant fish cmGS1 (84% amino acid identity) than to cmGS2 (72%). Among the three transcripts, variants 2 and 3 encoded the same GS molecule, while variant 1 contained the insertion of 95 nucleotides in the N-terminal region; the insertion position was 10 nucleotides prior to the ATG start codon of variants 2 and 3. This insertion resulted in the occurrence of additional ATG start sites 29 amino acids prior to the start codon of the variants 2 and 3. The N-terminally extended portion of variant 1 was predicted as a mitochondria targeting signal (MTS). Meanwhile, the amino acid sequence deduced from other two variants did not have the MTS (Fig. 17B). The probabilities of MTS calculated by MitoProt software were 0.94 for variant 1, while 0.44 for variants 2 and 3.

Different from elephant fish, in which two ARG genes (cmARG1 and cmARG2) exist, only a single gene transcript for ARG was found (Fig. 18). The deduced amino acid sequence of catshark stARG showed higher similarity to elephant fish cmARG2 (75%) than to cmARG1 (66%).

Expression levels of mRNAs encoding OUC enzymes during development

For developmental studies of fishes, elongation factor 1α (EF1α) has been

preferably used as an internal control for qPCR, because the levels of EF1α mRNA are relatively constant throughout development (McCurley and Gallard, 2008; Miyanishi et al., 2012; Takagi et al., 2012; Chapter 1). However, in catshark, expression of EF1α mRNA in the YSM was considerably lower than that in the embryo (Fig. 19B). Meanwhile, expression levels of stACTB were nearly identical between the embryos and the YSM (Fig. 19A). Therefore, stACTB was used as an internal control of the qPCR experiments in this study.

Figure 20 shows expression of GS, CPSIII, OTC, and ARG mRNAs in the developing embryos and the YSM from stage 20 to 33. The mRNA levels of all four enzymes relative to stACTB mRNA were kept very low in embryos until stage 31, and thereafter began to increase (Fig. 20). The expression levels of GS, OTC and ARG mRNAs in the YSM were also low until stage 31, but the levels in YSM were consistently higher than those in embryos (Fig. 20A, C, D). Meanwhile, the expression of the rate-limiting CPSIII mRNA was abundantly expressed in the YSM, and the levels in YSM were 3.1 to 12.6-folds higher than those in embryonic body from stage 20 to 32. The expression of CPSIII mRNA in the YSM gradually increased until stage 31 and thereafter decreased rapidly (Fig. 20B).

Since the masses of embryonic body and yolk were increased and decreased, respectively, during development, total amount of mRNA was then calculated for the embryonic body and the YSM as shown in Fig. 21. The developmental change in stCPSIII mRNA abundance in the YSM was the same pattern as the relative expression; the expression of stCPSIII mRNA was increased during the early developmental period, retained high levels until stage 31, and then decreased rapidly after stage 32 (Figs 20B and 21B). Meanwhile, the expression of stCPSIII mRNA in the embryonic body was gradually increased during the early to middle stages of development, and after stage 32, the levels in the embryonic body

were higher than those in the YSM. Similar developmental patterns in expression levels in the YSM and embryonic body were also seen for other enzyme mRNAs (Fig. 21).

For embryos from stage 32 to hatching, embryonic tissues (brain, liver, gill, muscle, kidney and gut) were dissected out, and distribution of OUC enzyme mRNAs was investigated. The CPSIII, OTC, and ARG mRNAs were primarily expressed in the embryonic liver and to a lesser extent in various tissues (Fig. 22B, C, D). The expression pattern of OUC accessory enzyme GS was broader, though the highest expression was observed in the liver (Fig. 22A).

Distribution of OUC enzyme mRNAs in the YSM

The YSM of catshark was easily separated into the two cell layers (outer cell layer which originated from ectoderm, and inner cell layer which originated from mesendoderm). Therefore, the two layers of YSM and embryonic body were separately sampled at stages 30 and 31, and mRNA levels of all OUC enzymes, including stASS and stASL, were examined. Consistent with the results of *in situ* hybridization for catshark CPSIII mRNA (see chapter 2-1, Fig. 13D), well-vascularized mesendodermal cell layer showed the highest mRNA levels for all OUC enzymes (Fig. 23). In particular, mRNA levels of stCPSIII, stARG and stASS in the inner layer were considerably higher than those in the embryonic body and ectodermal cell layer (Fig. 23B, D, E).

Activities of OUC enzymes in the YSM and embryonic body

The activities of OUC enzymes in the developing embryos were measured at stages 28, 30 and 32. By taking sample weight into account, total activities (μmols/min) in the YSM and the embryonic body were calculated. Both the

developing embryo and the YSM exhibited the activities for all OUC enzymes (CPSIII, OTC, ARG and GS) at all developmental stages examined (Fig. 24). At stages 28 and 30, activities of GS and ARG in the YSM were significantly higher than those in the embryonic body. The activities of rate-limiting CPSIII at stages 28 and 30 were very low both in the YSM and embryonic body; about half of samples were under detectable levels (one embryo and three YSM samples at stage 28, and four embryos and two YSM samples at stage 30). At stage 32, all enzyme activities both in the YSM and the embryonic body tended to increase; the increases were conspicuous for GS, CPSIII and OTC activities in the embryonic body. Therefore, those enzyme activities in the embryonic body were significantly higher than those in the YSM at stage 32, whereas ARG activity was higher in the YSM than in the embryo at all developmental stages examined (Fig. 24).

As investigated for mRNA expressions, relative activities (μmols/min/g) of OUC enzymes in the embryos and the inner and outer layers of YSM were examined at stage 32. Consistent with the mRNA expression data, the mesendodermal layer of catshark YSM showed the highest activities of GS, OTC and ARG (Fig. 25A, C, D). Meanwhile, CPSIII activities were low in all regions, and no significant difference in CPSIII activities was found among the three tissues (Fig. 25B).

Development of embryonic liver

Since the liver is the major organ for urea production in the embryonic body, changes in the liver mass were analyzed by histological observation. The liver mass was exponentially increased from stage 23 to 32 (Fig. 26), in parallel with the increase in the weight of embryos shown in Table 7.

DISCUSSION

In this chapter, I investigated urea production in developing embryos of catshark, and confirmed that catshark embryos conduct urea-based osmoregulation for adaptation to the high-osmolality marine environment. As I discovered in holocephalan elephant fish embryos, the YSM has the ability to synthesize urea also in catshark, and its contribution appears to be greater than the embryonic body until stage 32. Therefore, it is most probable that, in oviparous marine cartilaginous fishes, the YSM is a principal organ for urea production during the early to middle developmental period, and that the important contribution of YSM to ureosmotic strategy is a common phenomenon in oviparous cartilaginous fish.

In the present study, urea concentrations in the embryonic body and yolk were investigated together with the intracapsular ionic and osmotic environments surrounding the embryos. In catshark, the pre-hatching (the early opening of an anterior part of the egg capsule) occurred around stage 31. My results clearly showed that there is no difference in osmolality and chloride concentration between the intracapsular environment and surrounding SW throughout the development in *S. torazame*, regardless of whether egg capsule is tightly closed or not. This result is consistent with my previous results in elephant fish eggs described in chapter 2-1 and with preceding results (Needham and Needham, 1930; Hornsey, 1978; Foulley and Mellinger, 1980), implying that the wall of the egg capsule is permeable to ions. The wall of the egg capsule appears to be a highly porous structure, and the pore radius was calculated to be 13.6 Å, which is much larger than a radius of urea (approximately 2.3 Å) (Hornsey, 1978). Therefore, although Evans (1981) reported lower ionic concentrations and osmolality inside the capsule in *Raja erinacea* than those of the surrounding SW, the wall of the egg capsule most probably does not form a barrier for ions and small molecules, including urea.

The high-osmolality intracapsular environment implies that embryos do osmoregulate by themselves throughout the development. In accordance with this idea, high concentrations of urea were found both in catshark embryos and yolks. The high levels of urea were found from stage 28, which is the first stage I could analyze; the levels were nearly identical to the plasma urea levels of adult fish, and were maintained throughout the development. These results are consistent with the previous reports. Needham and Needham (1930) showed that the embryonic system (embryo plus yolk) contains considerable amount of urea throughout its development in *S. canicula*. Read (1968b) confirmed the findings by Needham and Needham by investigating various sizes of embryos, and further demonstrated that urea concentration in embryos and yolks of skate *R. binoculata* did not change significantly during development.

In *S. torazame*, the urea levels in yolks were 1.5 times lower than those in embryos at each developmental stage. A similar result was obtained in *R. binoculata* (Read, 1968b). In *R. binoculata*, water content in embryos is reported to be 1.5 times higher than that in yolks; when values for urea concentration in the embryos and yolks were corrected for their water contents, the resulting values for urea concentration were nearly equal between embryos and yolks (Read, 1968b). In the present study, the high levels of urea (221.09 mM) were detected also in the yolks of undeveloped eggs (Table 8), and the urea concentration in yolks did not change throughout the development, even after decreases in the yolk volume. Therefore, these results imply that there is no specific mechanism absorbing urea from the yolk for osmoregulation of the embryo. In other words, embryos must synthesize urea by themselves to maintain a high concentration of urea in the embryonic body during development. Needham and Needham (1930) and Read (1968b) demonstrated that the total amount of urea per embryonic system (embryo

plus yolk) markedly increased during development, suggesting a production of urea by the developing embryos.

In accord with the results of elephant fish embryo, the YSM of catshark embryo exhibited mRNA expression and activities of OUC enzymes. During the early to middle phases of development, the expression of rate-limiting CPSIII, OTC and ARG mRNAs in the YSM were considerably higher than those in the embryonic body. The high expression of OUC enzyme mRNAs was consistent with the results of OUC enzyme activities; the activities of GS and ARG in the YSM were significantly higher than those in the embryonic body. On the other hand, no difference in CPSIII and OTC activities was observed between the YSM and the embryonic body at stages 28 and 30. This is probably partly due to the low enzyme activities of OTC and rate-limiting CPSIII in both the YSM and the embryonic body. Indeed, five samples out of twelve were lower than the minimum detectable level of CPSIII activity both for the YSM and embryonic body.

After stage 32, in which the external gill filaments reach the maximum length and begin shortening, the amounts of OUC enzyme mRNAs were rapidly decreased in the YSM, while those in the embryonic body increased. Concomitant with the increases in mRNA expression, the activities of all four enzymes were conspicuously increased in the embryonic body of stage 32, and the levels of GS, CPSIII and OTC were significantly higher than those in the YSM. These results confirm my finding in elephant fish development, and imply that a distributional shift of the urea production site from the YSM to the embryonic body is the common phenomenon in development of oviparous cartilaginous fishes.

Tissue distribution analysis of OUC enzyme mRNAs showed that the liver is the primary organ for urea production in the embryonic body, as already reported in adult fish (Anderson et al., 2005; Chapters 1 and 2-1). Therefore, the changes in

liver volume were examined during the development of catshark embryo. A hepatic bud was first observed at stage 21, and the liver mass was exponentially increased from stages 23 to 32. However, both the mRNA levels and activities of OUC enzymes remained low in catshark embryonic body until stage 32, implying that the embryonic liver is not fully functional before stage 32. From stages 32 to 33, the mRNA expression of four OUC enzymes was markedly increased, and the concomitant increases in enzyme activities were observed in the embryo at stage 32. Therefore, the embryonic liver seems to be in the growing phase until stage 32 and, after stage 32, the embryonic liver most probably starts to function as the primary organ for urea production.

The YSM of oviparous cartilaginous fishes are composed of an ectodermal outer layer and a highly-vascularized mesendodermal inner layer (Lechenault et al., 1993). The tissue distribution analysis of all OUC enzymes, including ASS and ASL, revealed that OUC enzymes are predominantly expressed in mesendodermal inner layer of the YSM, supporting the previous results of *in situ* hybridization (see chapter 2-1). These results clearly showed that the mesendodermal layer contributes to urea production in the YSM. The urea produced in the mesendodermal layer of YSM is presumably transported through the blood circulation connecting between the yolk sac membrane and the embryonic body, and supplies urea to the developing embryos. Lechenault et al. (1993) suggested that gas exchange is a primary role of the extraembryonic YSM particularly before the pre-hatching period. Before development of the external gills, the expanded vitelline blood circulation on the surface of mesendodermal layer could cover for undeveloped gills. Therefore, it is obvious that the YSM essentially contributes to embryonic homeostasis during the early developmental period of oviparous cartilaginous fishes.

In summary, I found in chapter 2 that embryos of elephant fish and catshark conduct urea-based osmoregulation for adaptation to the high-salinity marine environment throughout their development. I discovered that the mesendodermal layer of extraembryonic YSM contributes importantly to urea production during early to middle phases of development, and that the distributional shift of the urea production site occurs from the YSM to the embryonic body during embryonic development in oviparous cartilaginous fishes. In the next chapter, I attempted to elucidate the regulatory mechanisms of embryonic urea production, by focusing on endocrine factors controlling the OUC enzymes.

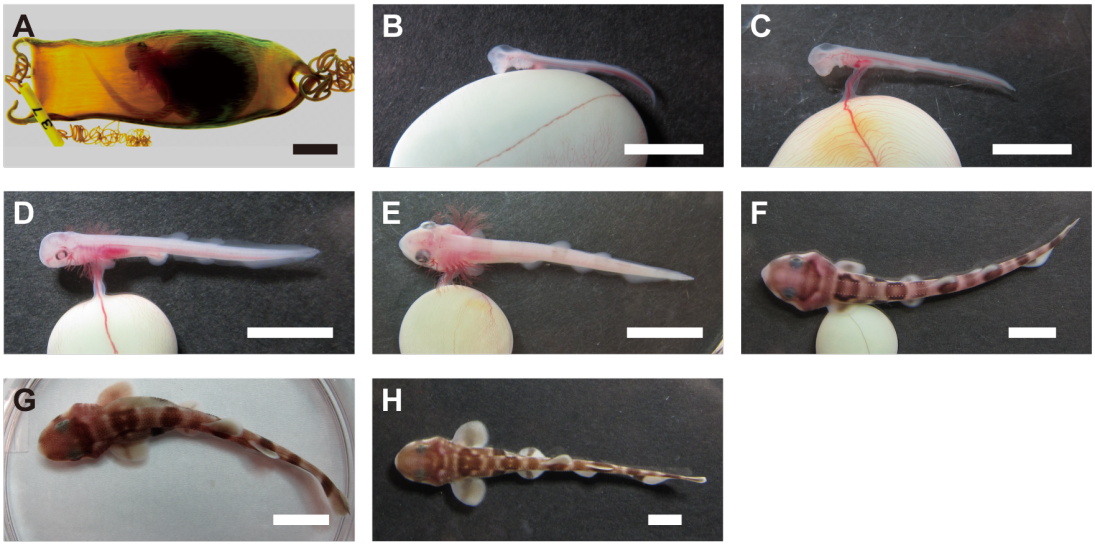

Figure 14. An egg capsule and representative developing embryos of catshark. Developmental stages were identified according to Ballard et al. (Ballard et al., 1993). (A) A semitransparent egg capsule, carrying the embryo of stage 32. (B) Embryo at stage 28. (C) Embryo at stage 30 showing faint black pigmentation around the lens of eye. (D) Stage 31. Pre-hatching occurs during this stage. The dorsal and anal fins appear. (E) Embryo at stage 32. In early period of this stage, external gill filaments reach the maximum length. (F) Stage 33. The external yolk sac is obviously shrinking, and yolk start moving into the internal yolk sac and the spiral intestine through the yolk stalk. (G) Stage 34. The external yolk sac can be observed as a tiny button-like structure. (H) Hatched fish. Scale bar = 1cm.

Table 7. Developmental changes in the weights of embryo and yolk

Stage	*N*	Embryo weight (mg)	Yolk weight (g)	*N*	External gill length (mm)
25	1	20	1.82	-	-
26	1	20	2.00	-	-
27	8	28.75 ± 5.48	2.03 ± 0.07	1	1.00
28	11	31.82 ± 3.77	1.90 ± 0.05	1	1.10
29	14	49.29 ± 4.50	1.97 ± 0.09	9	1.77 ± 0.20
30	27	73.33 ± 3.70	1.87 ± 0.05	14	2.84 ± 0.19
31	40	186.25 ± 8.78	1.78 ± 0.06	18	4.48 ± 0.21
32	51	431.96 ± 19.50	1.66 ± 0.05	8	4.79 ± 0.38
33	20	1272.27 ± 104.85	0.85 ± 0.11	3	2.96 ± 0.43
34	12	2605.00 ± 78.48	-	-	-
Hatched	7	3482.72 ± 385.99	-	-	-

Note. At stage 34, all yolk was transferred into the internal yolk sac, and the external yolk sac disappeared. The external gill filaments appeared from stage 27, reached maximum size at stage 32, and were regressed before stage 34. Values are presented as means ± SEM.

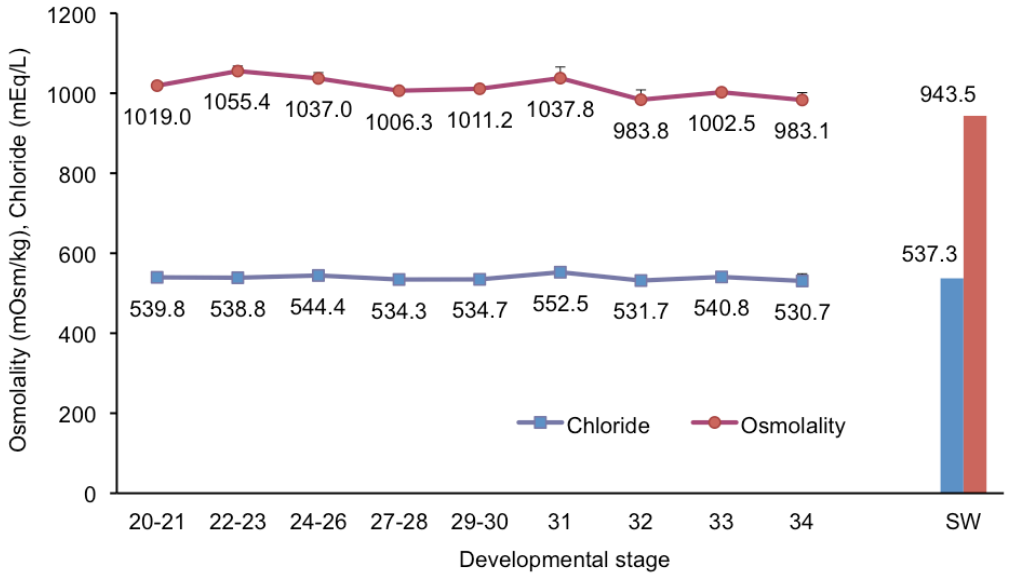

Figure 15. Chloride concentration and osmolality of intracapsular fluid of catashark egg capsules and seawater (SW). Values are presented as means ± SEM. Mean values were provided for each developmental stage and SW sample. The number of samples was as follows: N = 1 (SW); 2 (Stage 33); 3 (32); 4 (20-21, 31, and 34); 5 (22-23 and 24-26); 6 (27-28 and 29-30).

Table 8. Developmental changes of urea concentration in catshark embryos and yolks

	Undeveloped	St. 28	St. 30	St. 31	St. 32	Adult plasma
Embryo	-	349.36 ± 10.20	351.63 ± 3.52	347.61 ± 5.08	347.53 ± 7.77	320.65 ± 5.06
Yolk	221.09 ± 2.15	232.56 ± 6.60	245.85 ± 7.64	242.34 ± 5.10	237.26 ± 4.40	-

Note. The extracts of embryo (w/v) and yolk samples (v/v) were obtained by procedures as described in the method section. Values are presented as means ± SEM. $N = 3$ (Undeveloped egg) or 4 (Adult plasma, embryos and yolks).

Table 9. Primer sets used in the present study

Target	Primer sets for molecular cloning (5' to 3')		Amplicon (bp)
stCPSIII	Sense:	GTNGARTTYGAYTGGTGYGCNGT	1055
	Antisense:	TTGGGNCCRAARCANGCNACYTC	
stOTC	Sense:	TNGSNATGATHTTYGARAARMG	754
	Antisense:	GTCCAYTTNCKRTTYTCNGCYTC	
stGS	Sense:	CACTGTGACTGTGTTAAGGCTG	401
	Antisense:	TTACAGGACTTGGGGGTG	
stARG	Sense:	TGGGTNGAYGCNCAYRCNGA	350
	Antisense:	GGRTCRAANGCMTCDATMTCRAARC	
stACTB	Sense:	ATGTGYAARGCNGGNTTYGCNGG	1040
	Antisense:	TAYTCYTGYTTRCTDATCCACAT	
Target	Primer sets for RACE PCR (5' to 3')		
stGS	3'RACE:	ACTTTAGCACAAAATCTATGCGG	
	5'RACE:	CCATAAGCTTTATCTGCACCAAC	
stARG	3'RACE:	CATCCTGAAAAACTTTGGCATC	
	5'RACE:	ATGACACTGGCTGTCCATG	
Target	Primer sets for real-time qPCR assay (5' to 3')		
stCPSIII	Sense:	CTCACGATGCGACAGAGATGA	71
	Antisense:	GACAACTGGGTGTTCCTGAGAGA	
stOTC	Sense:	GGGCTTTGAACCAGATTCCA	70
	Antisense:	CATTTTGGTCCCACACTTTTCAG	
stGS	Sense:	GAGAGTCAGTCCGCTGCAAGA	81
	Antisense:	CATCAAAATTCCATTCTGGGAGAT	
stASS	Sense:	CCCTTGGAGTATGGATGCTAAC	112
	Antisense:	TGCTGGGTCAACTGTCATC	
stASL	Sense:	CCGATAGTCTGGAACTGATTCG	93
	Antisense:	ATGTGCTTGGCAGACCTTTA	
stARG	Sense:	GCTTGGAGGAGACCACAGTTTAG	212
	Antisense:	GAGTGATCCAAGAAAATCCAGG	
stACTB	Sense:	CCTGGCATTGCAGACCGTAT	74
	Antisense:	GCAATGATCTTGATTTTCATGGTACT	

Mixed bases: N, A/T/G/C; D, A/G/T; R, A/G; Y, C/T; S, C/G; H, A/C/T; M, A/C; K, G/T.

(A) Catshark CPSIII

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   1: GTAGAGTTTGATTGGTGTGCGGTCTCCAGCATCCGGACCCTGAGGCAGCTTGGGAAGAAG
  61: ACAGTGGTTGTAAACCATAACCCAGAGACTGTGAGCACTGATTTTGATGAGTGTGACAGG
 121: CTTTACTTTGAGGAGCTCTCGCTGGAAAGGATCCTGGATATCTATGAACAGGAGGGTTGC
 181: AAGGGGTGTATTATTTCTGTCGGTGGACAGATTCCAAACAACCTGGCTGTTCCCTTGTCC
 241: AAGTATGGTGTGAACATTCTGGGCACCAATCCAATGCAGATTGACCGCGCTGAGGATCGT
 301: GCTGTCTTCTCCGGGGTACTCGATGAGCTCCAGATTGCTCAGGCTTCGTGGATGGCTGTC
 361: AATTCTTTGGATGATGCACTTTCATTTGCTGACAATGTGGGCTACCCCTGCTTGCTGAGG
 421: CCATCTTATGTCCTAAGTGGTTCAGCGATGAATGTGGCTCACGATGCGACAGAGATGAAG
 481: AAATTCCTTGCGGAAGCGGCCCGTGTCTCTCAGGAACACCCAGTTGTCCTCACCAAATTC
 541: ATTGAAGGAGCACGGGAGGTTGAAGTTGATGCAGTTTCAAAGGAGGGAAAAGTGCTGGCA
 601: CACGCGATCACTGAGCATGTGGAAGATGCAGGTGTTCATTCTGGAGATGCAACATTGATT
 661: CTCCCAACACAGACCATCAGCCAAGGAGCCTTGGAGAAGGTGAAAATTGCAACCAGGAAG
 721: ATATCCAGAGCCTTCGAGATTTCTGGCCCTTTTAATGTCCAGTTTCTTGTGAAGGGAAAT
 781: GATGTAATGGTGATCGAATGTAACCTCCGAGCATCCAGGTCCTGCCCCTTTGTCTCAAAG
 841: ACCATTGGAGTTGATCTCATCAATGTGGCTACACGAGTGATGATCGGAGAAAGCATCGAT
 901: GAGGCGCTCCTCCCCTCACTGGAAAACCCCATCATACCTACTGAATATGTGGGCATCAAG
 961: GCTCCCATGTTTTCTTGGCCACGACTGAGGGATGCAGACCCTATTCTACGCTGTGAAATG
1021: GCATCAACTGGAGAAGTCGCATGCTTCGGCCCCAA   1055

   1: VEFDWCAVSSIRTLRQLGKKTVVVNHNPETVSTDFDECDRLYFEELSLERILDIYEQEGC
  61: KGCIISVGGQIPNNLAVPLSKYGVNILGTNPMQIDRAEDRAVFSGVLDELQIAQASWMAV
 121: NSLDDALSFADNVGYPCLLRPSYVLSGSAMNVAHDATEMKKFLAEAARVSQEHPVVLTKF
 181: IEGAREVEVDAVSKEGKVLAHAITEHVEDAGVHSGDATLILPTQTISQGALEKVKIATRK
 241: ISRAFEISGPFNVQFLVKGNDVMVIECNLRASRSCPFVSKTIGVDLINVATRVMIGESID
 301: EALLPSLENPIIPTEYVGIKAPMFSWPRLRDADPILRCEMASTGEVACFGP   351
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(B) Catshark OTC

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   1: TTGGAATGATTTTTGAAAAGCGGAGCACCAGAACAAGAATTTCTACAGAAACAGGGTTTG
  61: CATTGCTTGGAGGGCACCCCTGCTTCCTTACAACACAAGATATTCATCTTGGGGTTAATG
 121: AGAATCGCAAAGACACAGCAAGGGTTTTGTCTGGGATGACTGACCTGATCCTCGCTAGGG
 181: TCTATAAACACTCTGATCTGGAACAGTTGGCAGAAGATGGTTCAATCCCTGTGATCAATG
 241: GATTGTCAGAGCTATATCATCCTGTCCAGATCCTGGCTGATTACCTCACACTTCAGGAAC
 301: ATTTTGGCTCACTGACTGGTCTAACTCTGTCTTGGATAGGAGATGGAAACAATGTGTTAC
 361: ACTCTTTCATGTTGAGTGCTCCTAAACTCGGAATGCATCTTCATGCTGCTACACCTAAGG
 421: GCTTTGAACCAGATTCCAATGTCACCGCGTATGCCAAGTCATTGTCTGAAAAGTGTGGGA
 481: CCAAAATGTTTTTCACTAACGATCCTCTGGAAGCAGCAAATGGAGCCAATGTTCTGGTAA
 541: CAGATACGTGGATCAGCATGGGGCAAGAAGAAGAGAAAGTGGAAAGGCTGAAAGCGTTTC
 601: ATGGTTATCAAATCACCATGAAGACCGGACAGTCAGCTGCACCCAACTGGATATTCTTGC
 661: ATTGTTTGCCACGAAAACCGGAGGAAGTTGATGATGAAGTATTTTATTCCCCGAGATCAC
 721: TGGTCTTCCAAGAAGCCGAAAACCGCAAATGGAC   753

   1: STRTRISTETGFALLGGHPCFLTTQDIHLGVNENRKDTARVLSGMTDLILARVYKHSDLE
  61: QLAEDGSIPVINGLSELYHPVQILADYLTLQEHFGSLTGLTLSWIGDGNNVLHSFMLSAP
 121: KLGMHLHAATPKGFEPDSNVTAYAKSLSEKCGTKMFFTNDPLEAANGANVLVTDTWISMG
 181: QEEEKVERLKAFHGYQITMKTGQSAAPNWIFLHCLPRKPEEVDDEVFYSPRSLVFQ   236
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(C) Catshark ASS

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   1: TAGCCTACAGTGGTGGATTGGACACCTCATGCATTCTGGTCTGGCTAAAGGAACAAGGAT
  61: ATGAAGTTATTGCATATCTGGCTAACGTGGGACAGGATGAAGATTTTGAAGCTGCTGAAA
 121: AGAAAGCAGTTTCTCTGGGAGCTAAAAAGGTTTTTATTGAAGATTTGCGAAAGGAATTTG
 181: TGGAAGAGTTCATCTGGCCATGGGTGCAGTCAAATGCTATTTATGAGGACCGATACTTCC
 241: TTGGCACGTCACTGGCTAGGCCCTGTATTGCTAGAGGCCAGGTTAAGATTGCAAAACAGG
 301: AAAAAGCTCAGTTTGTGTCCCATGGAGCCACAGGAAAGGGAAATGATCAGATTCGGTTTG
 361: AGTTGACTTGCTATGCACTATATCCTGAAGTCAAGATCATCTCCCCATGGAGATTACCAG
 421: AGTTCTACAATCGATTCATGGGACGCACTGATTTGATGGAATATGCAAAGGCACATGGCA
 481: TTCCTGTTCCTGTCACTCCCAAAAGCCCTTGGAGTATGGATGCTAACATTATGCATATCA
 541: GCTATGAATCTGGGATCCTGGAAAACCCCAAGAATCACGCACCGGATGGTATGTTCATGA
 601: TGACAGTTGACCCAGCAAAGTCTCCAAACACTCCGGACGAATTGGAGATTGAGTTCAAAA
 661: ATGGTGTCCCTGTCAAGGTCACCAACCTGGGTGACAAGACCACTCGGAGCTCGGCCCTTG
 721: AACTGTTCATGTAYGTCAATGAAATTGGGGGTAAGCATGGGGTTGGACGTATAGACATCG
 781: TGGAGAATCGCTTCATTGGCATGAAATCACGCGGTATCTATGAGACCCCAGGTGGGACAA
 841: TTCTGTATCAAGCTCATGTGGACATTGAGGCCTTCACCATGGACAAGGAAGTGAGGAAAA
 901: TCAAGCAGCAACTGTCACTGAAGTTTTCCGAACAGCTGTACAATGGATTTTGGGACAGCC
 961: CTGAGTGTGCATTTGCCCGGCACTGCATCAACAAATCACAGGAGCATGTCG   1011

   1: AYSGGLDTSCILVWLKEQGYEVIAYLANVGQDEDFEAAEKKAVSLGAKKVFIEDLRKEFV
  61: EEFIWPWVQSNAIYEDRYFLGTSLARPCIARGQVKIAKQEKAQFVSHGATGKGNDQIRFE
 121: LTCYALYPEVKIISPWRLPEFYNRFMGRTDLMEYAKAHGIPVPVTPKSPWSMDANIMHIS
 181: YESGILENPKNHAPDGMFMMTVDPAKSPNTPDELEIEFKNGVPVKVTNLGDKTTRSSALE
 241: LFMXVNEIGGKHGVGRIDIVENRFIGMKSRGIYETPGGTILYQAHVDIEAFTMDKEVRKI
 301: KQQLSLKFSEQLYNGFWDSPECAFARHCINKSQEHV   336
```

(D) Catshark ASL

```
   1: TGGACAAGATTGCCGAAGAATGGACAAATGGCACTTTTGAGATTAAAGAAGGAGATGAGG
  61: ACGTTCACACAGCCCATGAACGAAGACTAAAGGAGTTGATTGGAGAAGCGGGAGGAAAAT
 121: TGCATACTGGCCGAAGCAGGAATGATCAGGTGGTAACTGACATGAGAATATGGCTGCAAG
 181: ATGAAATCTCCGTTCTGATGGGTCACTTGCAGCAGTTAATAAAAACGATGGTTGAAAGAG
 241: CAGCAGCGGAGATTCATATCCTTTTCCCTGGATATACTCATATGCAAAGAGCACAACCAA
 301: TTCGATGGAGTCATTGGATACTGAGCCATGCTGCTGCCTTGACTCGTGATGCTGAAAGAC
 361: TGGACGAAATTAAGAAACGAACCAATATCCTGCCATTGGGAAGTGGTGCTATTGCTGGAA
 421: ATCCCTTTAAGATTGACAGAGACTTCCTCCGTAAGGAGCTGCAATTTGATGCCATTTCTT
 481: TAAACAGCATGGATGCAACCAGTGGACGAGACTTTGTTGCTGAGATCATGTTCTGGTGCT
 541: CCTTGTGTATGATCCACCTGAGTAAGATATCCGAGGATCTAATCATTTACAGCACCGCGG
 601: AATTTGGATTCGTAAGCCTGTCTGATGCCTACAGCACTGGGAGTAGCCTGATGCCCCAGA
 661: AGAAGAATGCCGATAGTCTGGAACTGATTCGGGGCAAGACTGGAAGTGTGTTTGGGCGGT
 721: TTTCTGGATTTTTGATGACTCTTAAAGGTCTGCCAAGCACATACAATAAGGATTTGCAGG
 781: AAGACAAGGAGGCAATGTTTGAAAGCATTGACACCGTCACTGCCGTACTCCAAGTGGCAT
 841: CAGGTGTAATATCTACATTAAAGGTTCATGAAGATGCCATGAAGAAGGCTTTGAGCCCAA
 901: ACATGCTGGCTACAGATCTGGCTTATTACTTGGTTCGCAAGAAGATGCCATTCAGGGAAG
 961: CCCACAGTGCTTCTGGAAGAGCTGTTCACTTGGCAGAAAAGAAAGGTATTGAAATGGATC
1021: AGCTCACCCTGGATGATTTCAAGACCATTAGTCCTCTGTTTGAGAGTGA   1070

   1: DKAWTNGTKGDDVHTAHRRKGAGGKHTGRSRNDVVTDMRWDSVMGHKTMVRAAAHGYTHM
  61: RARWSHWSHAAATRDARDKKRTNGSGAAGNKDRDRKDASNSMDATSGRDVAMWCSCMHSK
 121: SDYSTAGVSSDAYSTGSSMKKNADSRGKTGSVGRSGMTKGSTYNKDDKAMSDTVTAVVAS
 181: GVSTKVHDAMKKASNMATDAYYVRKKMRAHSASGRAVHAKKGMDTDDKTSS   231
```

Figure 16. The partial cDNA and deduced amino acid sequences of stCPSIII (A), stOTC (B), stASS (C), and stASL (D). Target positions of oligonucleotide primers were marked with gray background. Numbers on the left side indicate the nucleotide number.

(A) 5' sequences of catshark stGS transcripts

```
variant1:  ATGGGGATTCTTCACTGTGACTGTGTTAAGGCTGTGATCT   40
variant2:  -----------TCACTGTGACTGTGTTAAGGCTGTGATCT   29
variant3:  -----------TCACTGTGACTGTGTTAAGGCTGTGATCT   29

           CAG-----------------------------------   43
           CAGCTGCTGTTGGAGTTCTGATCCAATTCAATATTCTGTA   69
           CAG-----------------------------------   32

           ----------------------TGCAGCTGAATGAACAA   60
           TGAAATGGAAAAGGAAGTATTTTTGCAGCTGAATGAACAA  109
           ----------------------TGCAGCTGAATGAACAA   49

           TCCTGAACTGGACAGAACAGAACAGAGAAGAGTATAGCCA  100
           TCCTGAACTGGACAGAACAGAACAGAGAAGAGTATAGCCA  149
           TCCTGAACTGGACAGAACAGAACAGAGAAGAGTATAGCCA   89

           AGCCTCTGTAGCAGGAAATCCGAGAGGAGGCGTTGGGCCA  140
           AGCCTCTGTAGCAGGAAATCCGAGAGGAGGCGTTGGGCCA  189
           AGCCTCTGTAGCAGGAAATCCGAGAGGAGGCGTTGGGCCA  129

           GCAAGTCTGTTTTTGGCGGAGGATGCGGATCTCACGCTGT  180
           GCAAG-------------------------------------  194
           GCAAG-------------------------------------  134

           TTCCTGTTTCTGGTAAAAAAATGCGGCAATATCAAGCCGG  220
           ----------------------------------------  194
           ----------------------------------------  134

           CGAATTGGCGGAACCAACATACTTACAAGATGGCCACTTC  260
           --------------------ACTTACAAGATGGCCACTTC  214
           --------------------ACTTACAAGATGGCCACTTC  154

           AGCCAGCGCCAATCTCAGCAAAATCGTCAAGAAGAATTAC  300
           AGCCAGCGCCAATCTCAGCAAAATCGTCAAGAAGAATTAC  254
           AGCCAGCGCCAATCTCAGCAAAATCGTCAAGAAGAATTAC  194
```

(B) N-terminus of the stGS

```
 variant  1   : MRISRCFLFLVKKCGNIKPANWRNQHTYKMATSASANLSKIVKKNYMELP 40
variants 2&3  : ----------------------------MATSASANLSKIVKKNYMELP 11

                QDGKVQAMYIWIDGTGESVRCKTRTLDKEPKAVSDLPEWNFDGSSTYQSE 80
                QDGKVQAMYIWIDGTGESVRCKTRTLDKEPKAVSDLPEWNFDGSSTYQSE 51
```

Figure 17. Alignments of 5' nucleotide sequences of three stGS transcripts (A), and deduced amino acid sequences of N-terminal regions (B). Identical nucleotides and amino acid residues are indicated with gray background. The putative initiation codons for variant 1 and 2&3 are indicated with red and black backgrounds, respectively.

Catshark ARG

```
ATGGGGGTTAAACTGGAGAGATGTCTTTACAAAGGGGATTTACGGAGCTCCTGCAGAAAC  :  60
GGCTCGGCTGGTTCCCCAAGCAGAGACGATTGGTGCATTCAGTGGGAATAATAGGAGCCC  : 120
CGTTCTCCTACGGCCAGAAACGGCGGGGAGTCGAAGGTGGGCCGCGAGCAATCCGGGACG  : 180
CCGGCTTGACTGAGAGGCTATCGGGTCTGGGCTGTAAAGTGTACGACTATGGAGACCTGA  : 240
GTTTCACCACTGTACCAAATGATACGCCGTACAACAACATTGTCCACCACCCAAGAACAG  : 300
TGGGTTTGGCCAACCAGTCATTAACTGAAGCAGTCAGCCGAGCAGTTGGTGATGGCCACC  : 360
TAAGCATTATGCTTGGAGGAGACCACAGTTTAGGAATTGGTTCGGTTCATGGGCACGCAC  : 420
AACAGCGACCTGATTTATGCGTCATATGGGTGGATGCGCATTGTGATCTAAACACACCTC  : 480
TTACTTCAATGTCTGGTAATCTACATGGACAGCCAGTGTCATTCCTTATAAGAGAGCTCC  : 540
AAGACAAGATACCATCACTTCCTGGATTTTCTTGGATCACTCCTTGCATCTCCGCCAAGG  : 600
ACTTGGTTTACATTGGACTACGGGATGTTGATCCTGCTGAATATTACATCCTGAAAAACT  : 660
TTGGCATCCGATACTTCTCTATGAAAGAGGTGGATCAACTCGGAATAAGGAAAGTAATGG  : 720
AAAGAACGTTTGATCATCTAATTGGAAAACAGAAAAGACCAGTGCACCTGAGTTTTGATG  : 780
TCGATGCTATTGACCCATCTTTAGCTCCTGCAACTGGCACTCCAGTGATAGGAGGACTAA  : 840
CTTACAGGGAAGGAATGTATATTGCAGAGGAAATACATAACACTGGAATGCTGTCTGCAA  : 900
TGGATCTGGTAGAAGTCAACCCCAAGCTTGCAGCTTCAGATGAGGAACTTAATGCGACAG  : 960
TAAACATTGCAGTGGATGTCATCTCCTTTTCTTTGGGACAGGCCCGAGAAGGATCCCATG  :1020
GCAAATGTGATGAACTACCGACACCCAGTTCAACATATGCTACAAATGAACAGACGCTAC  :1080
GGCTTTAGGACCATTTTGTTGAAGAAAGGAAGCAGTACAAATTCATATTCCAGAACTGAA  :1140
ATTTAAAGGAATAGTACTTTGTTTCACATACACTTTATCTTTGGGACACCGTGTTCTCAG  :1200
CCCCTTCATCTATATCCCCCTTTGTTCACAAGGTAATGTGGGCAACAGCCACATTTTCCT  :1260
ATACATTCCAGTGTGAAGAATAGGATAATTTCCCTATTCAACTAGCTGGAGATAAATTTG  :1320
AATAAGCCATCAAAACAGCTCTTTTCTTGGCCAGGGAATCATGAGAACGTTAAGAAGCTT  :1380
TGTGCTTGTTTGCATAAAAATCCTTTAAGATTGTTGGAGGGATAAATCTGTATACTGTTA  :1440
ATAATGTATATTAAATTTTCCCTCCAGATATAAATAACTTATAGGACATTTGTTAAATAT  :1500
CAAATGACTTTATTAAAAGTTGTGCACATTACTACAATGTAGTTATTAGCAAAGTTTTAA  :1560
GGGACTGATCAAATGCAACTTGATTTGTTAAGTTTCTTCATTTTGCTCAAAGATACTTCA  :1620
TCAGCTGCAAGACCCATCAAACCACAGGGACGTCGTCTAACCAGGTTAAGTACGTGATGA  :1680
CGAATGGAATGGTTCAGCAATAAAAGTACTTTAATACAC(poly A)                :1719

MSLQRGFTELLQKRLGWFPKQRRLVHSVGIIGAPFSYGQKRRGVEGGPRAIRDAGLTERL  :  60
SGLGCKVYDYGDLSFTTVPNDTPYNNIVHHPRTVGLANQSLTEAVSRAVGDGHLSIMLGG  : 120
DHSLGIGSVHGHAQQRPDLCVIWVDAHCDLNTPLTSMSGNLHGQPVSFLIRELQDKIPSL  : 180
PGFSWITPCISAKDLVYIGLRDVDPAEYYILKNFGIRYFSMKEVDQLGIRKVMERTFDHL  : 240
IGKQKRPVHLSFDVDAIDPSLAPATGTPVIGGLTYREGMYIAEEIHNTGMLSAMDLVEVN  : 300
PKLAASDEELNATVNIAVDVISFSLGQAREGSHGKCDELPTPSSTYATNEQTLRL       : 355
```

Figure 18. The full-length cDNA and deduced amino acid sequences of stARG. The coding region and the putative initiation codon "ATG" of stARG were shown with black and red backgrounds, respectively.

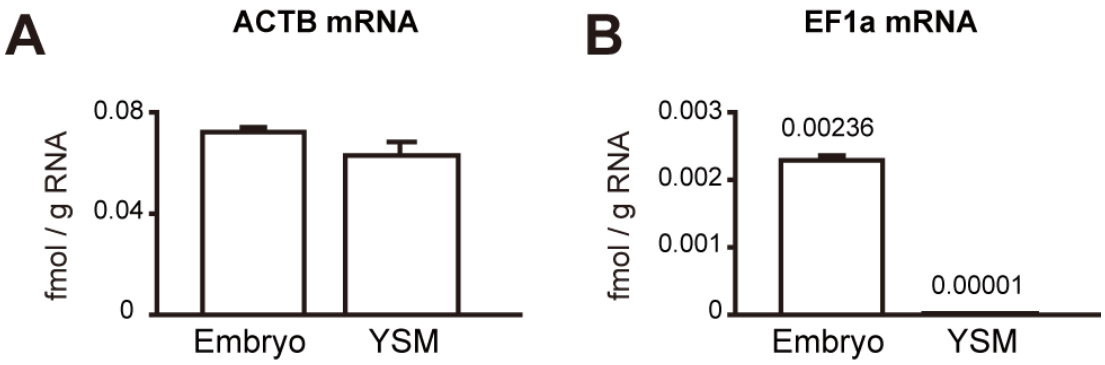

Figure 19. The expression of two commonly used house-keeping genes, stACTB (A) and elongation factor 1α (stEF1α) (B) in the YSM and embryo of stages 27 - 30 (N = 16). All data are presented as means ± SEM. The mean values are provided above the columns in B.

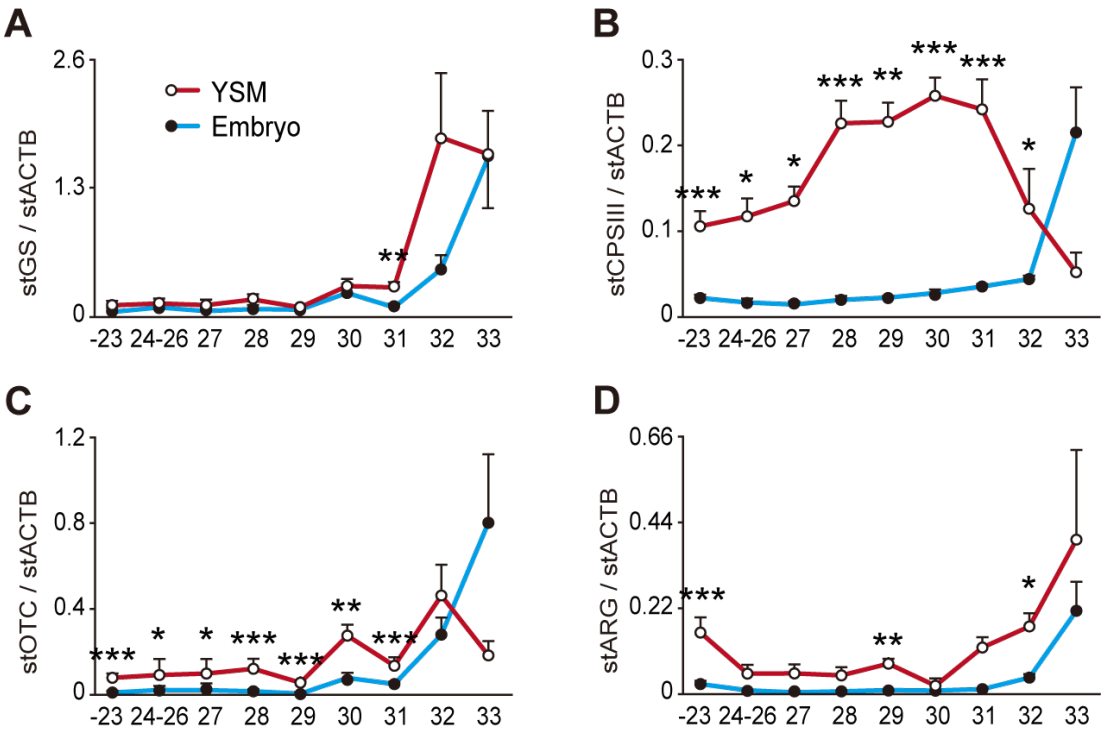

Figure 20. Developmental changes in mRNA levels of catshark stGS (A), stCPSIII (B), stOTC (C), and stARG (D) from stage 20 to 33. The mRNA levels of embryos from stage 20 to 23 and from 24 to 26 were grouped as "-23" and "24-26", respectively. The mRNA expression of OUC enzymes was normalized with stACTB mRNA levels. All data are presented as means ± SEM. $N = 6$ (Stages -23, 29, 30, 31); 4 (24-26, 27); 9 (28); 5 (32,33). Statistically significant differences between the YSM and the embryo are shown with asterisks. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

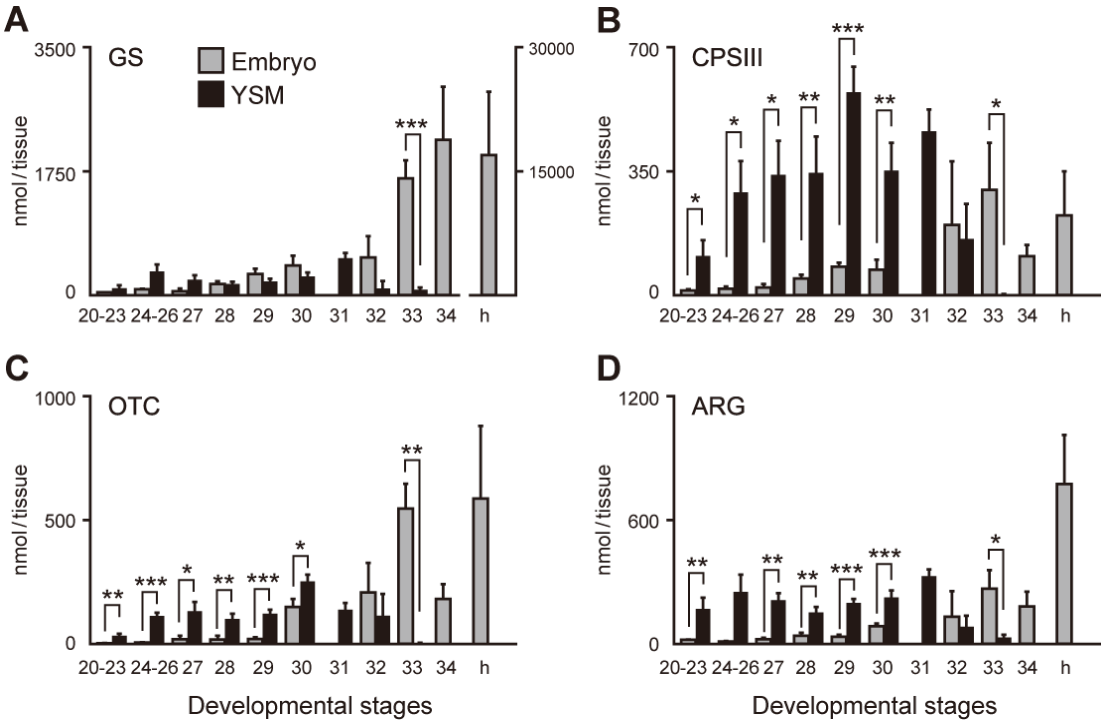

Figure 21. Developmental changes in mRNA abundance of catshark stGS (A), stCPSIII (B), stOTC (C), and stARG (D) from stage 20 to hatching. The grouping of the developmental stages was conducted as described in Figure 20. All data are presented as means ± SEM. $N = 6$ (Stages -23, 29, 30, 31); 4 (24-26, 27); 9 (28); 5 (32,33). Statistically significant differences are shown with asterisks. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. h, hatched fish.

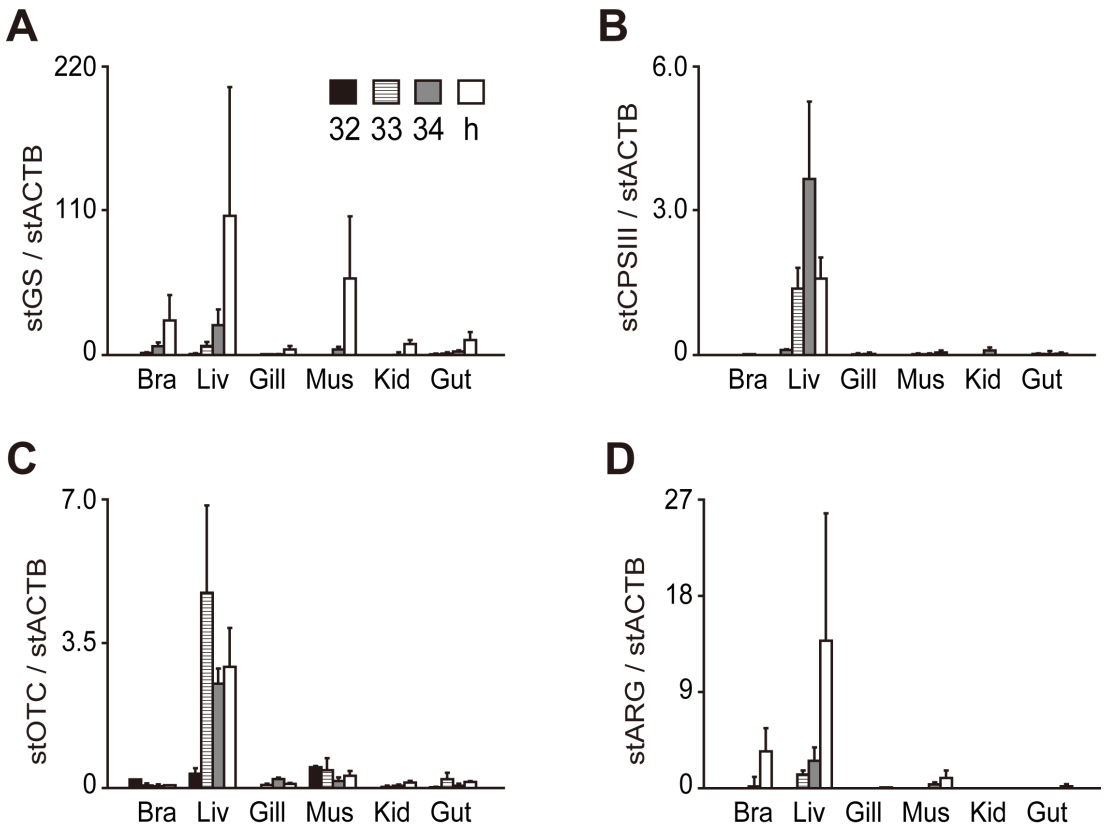

Figure 22. Tissue distribution of the OUC enzyme mRNAs in the embryonic tissues during the late developmental period, from stage 32 to hatching. stGS (A), stCPSIII (B), stOTC (C), and stARG (D). All data are presented as means ± SEM ($N = 5$). Bra, Brain; Liv, Liver; Mus, Muscle; Kid, Kidney.

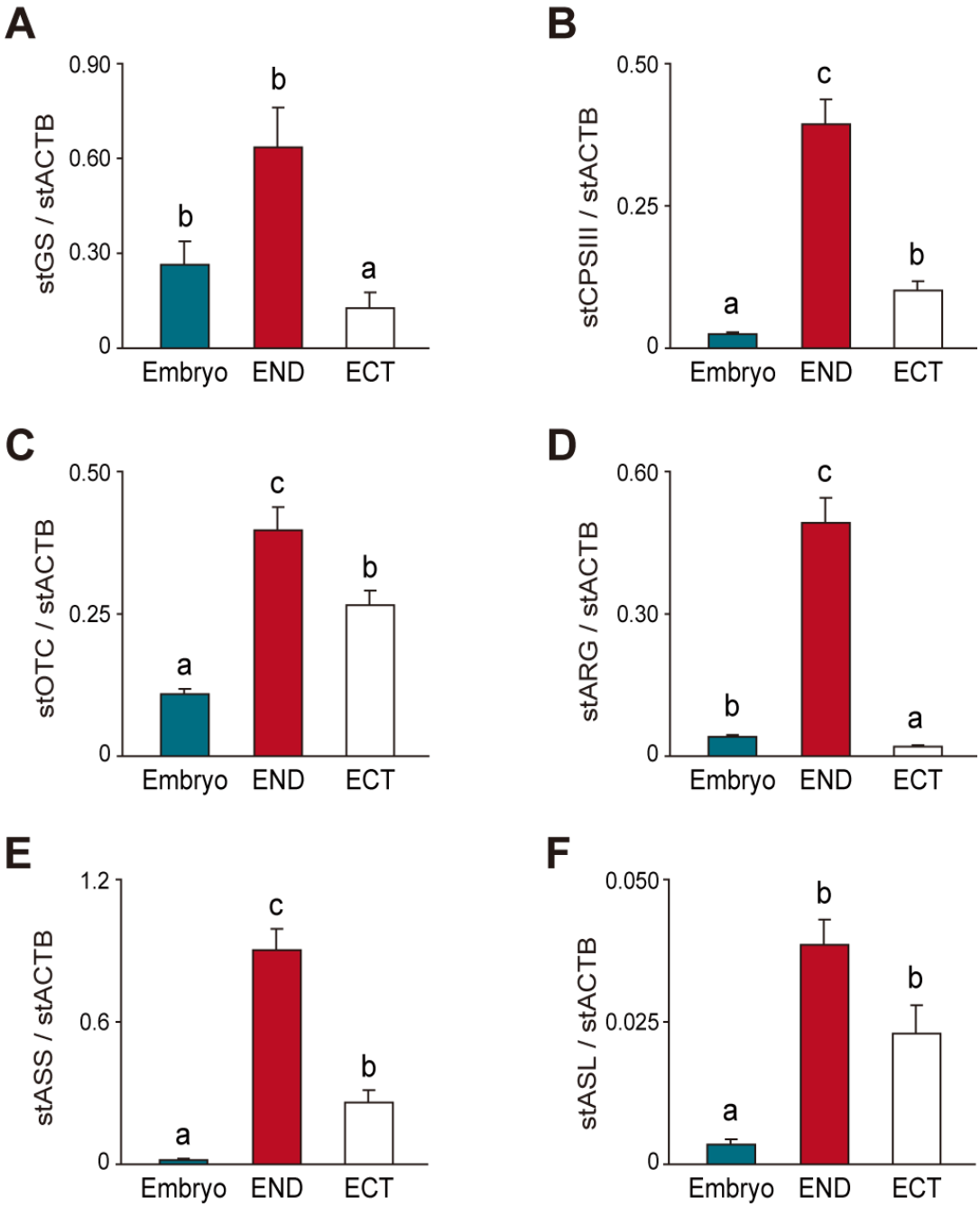

Figure 23. The mRNA levels of OUC enzymes in the embryos and extra-embryonic tissues of the developing embryos at stages 30 to 31 (N = 12). stGS (A), stCPSIII (B), stOTC (C), stARG (D), stASS (E) and stASL (F). END, mesendodermal layer; ECT, ectodermal layer of the YSM. All data are presented as means ± SEM. Different letters indicate significant differences between the samples ($P < 0.05$).

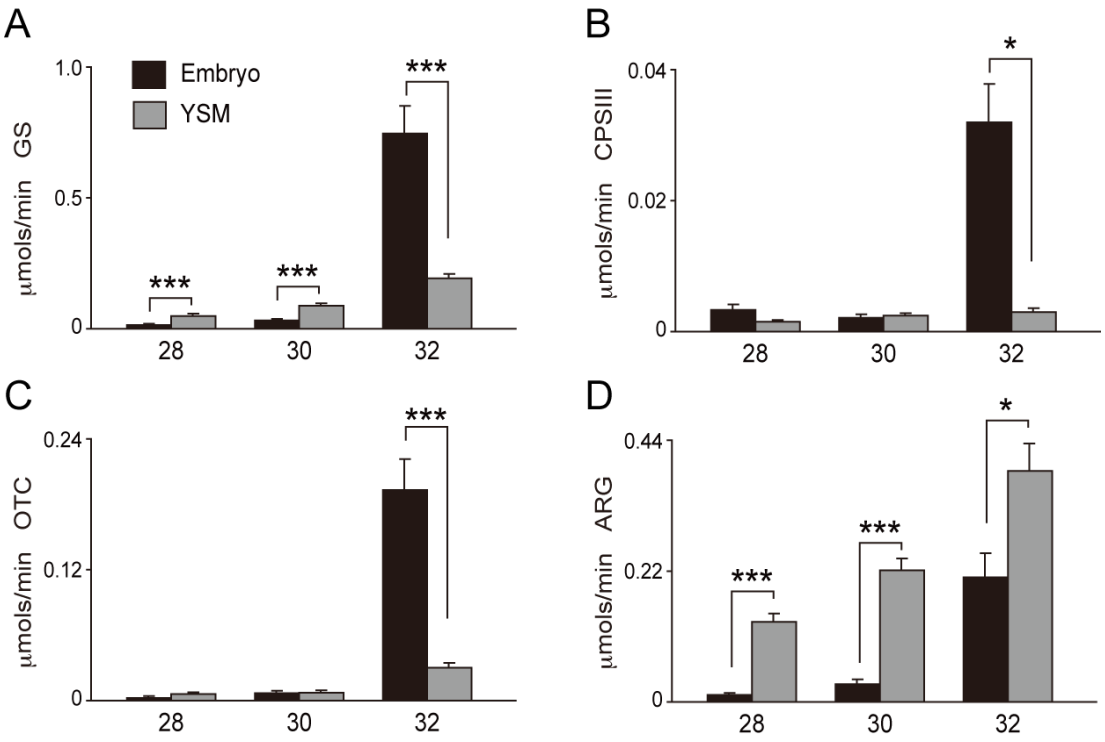

Figure 24. Developmental changes of the GS, CPSIII, OTC and ARG activities in the embryo and the YSM. All data are presented as means ± SEM ($N = 6$). *$P <$ 0.05, ***$P < 0.001$).

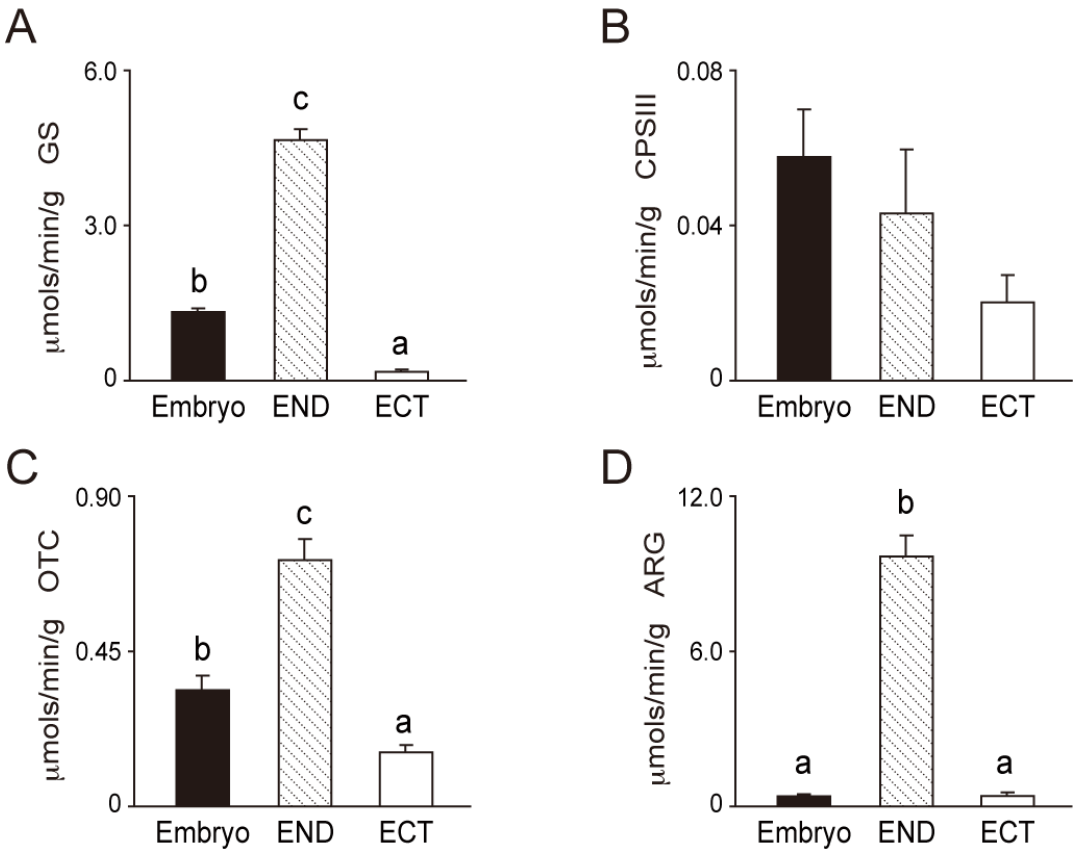

Figure 25. Enzyme activities of GS, CPSIII, OTC and ARG in the embryos and extra-embryonic tissues at stage 32. END, mesendodermal layer; ECT, ectodermal layer of the YSM. All data are presented as means ± SEM ($N = 6$). Different letters indicate significant differences between the samples ($P < 0.05$).

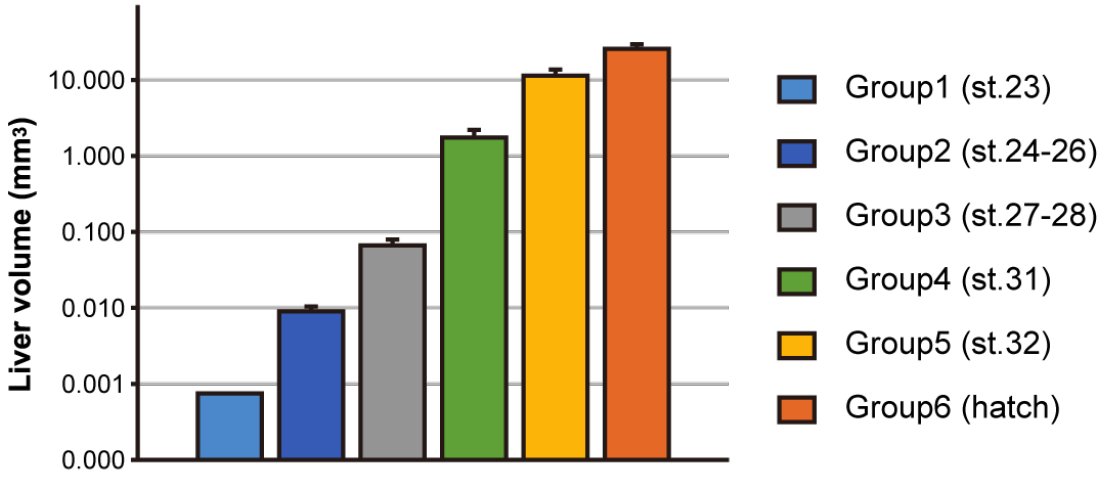

Figure 26. Changes in the liver volume from stage 23 to hatched fish. N = 1 (Group 1) and 3 (Group 2 to 6). Data are presented as means ± SEM, except for group 1.

Chapter 3

Endocrine control of urea production in the developing embryo of cloudy catshark (*Scyliorhinus torazame*): with special reference to glucocorticoids

ABSTRACT

In chapter 2, I found considerable contribution of the extra-embryonic yolk sac membrane (YSM) to embryonic osmoregulation of oviparous cartilaginous fish. As the development proceeded, the mRNA levels of ornithine urea cycle enzymes declined in the YSM, while increasing in the embryonic liver. The observed shift of urea-producing site indicates the existence of upstream factors regulating urea production in the YSM and the embryonic body. Thus, I performed transcriptome analysis on the catshark embryo and YSM, and focused on two nuclear receptors, glucocorticoid and mineralocorticoid receptors (GR and MR). The full-length coding sequences of GRs and MRs were determined in catshark and elephant fish, and the ligand selectivities and sensitivities of GRs and MRs were examined by an *in vitro* reporter assay. The transactivation activities of cartilaginous fish GRs were induced by corticosterone more efficiently than by 1α-hydroxycorticosterone, a principal circulating corticosteroid. The *in vivo* administration of corticosterone upregulated the stARG mRNA levels in the mesendodermal layer of YSM, while the increase was restored by co-administration with RU486. The corticosterone treatment also significantly increased stGS mRNA levels in the embryonic body and the ectodermal layer of YSM, while reduced stGS mRNA levels in the mesendodermal layer. Taken together, my findings suggest the importance of glucocorticoids and their receptors in the regulation of urea production in developing embryos.

INTRODUCTION

It is now well recognized that marine cartilaginous fish adopt a ureosmotic strategy for adaptation to a high-salinity marine environment. To this end, various organs work in a coordinated manner by producing (liver, muscle, intestine, kidney) and retaining (gill, kidney) urea, ensuring that cartilaginous fish maintain high levels of urea in their body (Hazon et al., 2003). In chapter 2, I further verified that embryos of oviparous cartilaginous fish also conduct urea-based osmoregulation even during the early developmental period, in which the above osmoregulatory organs are not fully developed or are not formed. I discovered that the extra-embryonic yolk sac membrane (YSM) contributes importantly to urea production during the early to middle phase of development. The OUC enzymes (glutamine synthetase (GS), carbamoyl phosphate synthetase III (CPSIII), ornithine transcarbamylase (OTC) and arginase (ARG)) were abundantly expressed in the mesendodermal layer of the YSM. Thereafter, the mRNA levels of OUC enzymes in the YSM declined during the late phase of development, while mRNA levels and activities of OUC enzymes markedly increased in the embryonic liver. These results imply a shift of the urea production site during embryonic development from the YSM to the embryonic liver, and thus an existence of upstream regulatory factors modulating the expression of OUC enzymes during the development (chapters 2-1 and 2-2; Takagi et al., 2014).

A number of endocrine factors have been implicated in regulation of vertebrate osmoregulation and urea production. In mammals, glucocorticoids induce increased expression and activities of OUC enzymes via glucocorticoid receptor (GR) in fetal rat liver (Gautier et al., 1977; Lamers and Mooren, 1981; Haggerty et al., 1983; Husson et al., 1983). Cortisol, one of the major glucocorticoids among tetrapods and teleost fishes, exerts crucial roles in

osmoregulation by regulating branchial ion uptake and secretion, and water absorption in intestine (Utida et al., 1972; McCormick et al., 2008; Kumai et al., 2012). Cartilaginous fishes possess a unique corticosteroid, 1α-hydroxycorticosterone (1α-OHB) (Idler and Truscott, 1966), and several studies reported the mineralocorticoid-like actions of 1α-OHB in elasmobranchs. For example, the surgical ablation of the interrenal gland, which is a homologous organ to the adrenal cortex, reduced sodium output and decreased Na^+/K^+-ATPase activity in the rectal gland of winter skate (*Leucoraja ocellata*) (Holt and Idler, 1975). To date, however, involvements of the 1α-OHB and other endogenous corticosteroids in urea synthesis have not been clarified in cartilaginous fishes.

The next-generation sequencing of transcripts (RNA-seq) is a powerful tool to find gene products exerting important function, and applications of the RNA-seq for unsequenced non-model organisms have also been reported (Emrich et al., 2007; Morozova et al., 2009; Mutz et al., 2013). In this chapter, I first conducted an RNA-seq analysis to find candidate genes regulating urea production and retention in the YSM and the embryonic body. Among a large number of genes found by the next generation analysis, I focused on glucocorticoid and mineralocorticoid receptors (GR and MR) because of the possible contribution to urea-based osmoregulation as mentioned above. I obtained full-length coding sequences of GR and MR of catshark and elephant fish, and investigated their ligand selectivity by gene reporter assay. Finally, effects of corticosteroids on expression of OUC enzymes in the YSM and the embryonic body were examined using an *in vivo* culture system.

MATERIALS AND METHODS

Animals and reagents

Catshark embryos were cultivated in a tank in the aquarium of AORI, and were sampled as described in chapter 2-2. All animal experiments were conducted according to the Guidelines for Care and Use of Animals approved by the committee of the University of Tokyo. Cortisol, corticosterone (B), 11-deoxycorticosterone (DOC), dextamethasone (DEX), 17β-estradiol (E2) and mifepristone (RU486) were obtained from Sigma (St. Louis, MO, USA), and 1α-hydroxycorticosterone (1α-OHB) was kindly gifted by Dr. Andrew N. Evans of University of Southern Mississippi and Dr. W. Gary Anderson of University of Manitoba. All reagents were dissolved in 100% ethanol (EtOH) (Wako).

Transcriptome analysis

The YSM and the embryonic body of catshark were sampled at stage 32 as described in chapter 2-2, quickly frozen in liquid nitrogen, and were stored at -80 ^{o}C. Total RNA of YSMs and embryonic bodies of two individuals were extracted with ISOGEN (Nippon Gene) as described in chapter 1. After purification with Oligotex^{TM}-dT30 <super> mRNA Purification Kit (TaKaRa), quality of RNA was assessed by bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Adaptor-ligated cDNA libraries were generated, and emulsion PCR was performed with GS Junior with Titanium cDNA Rapid Library Preparation Method (Roche). High-throughput sequencing was conducted on PCR products by using a 454 GS junior system (Roche). The data were assembled by GS De Novo assembler v. 2.7 (Roche), using the default settings. In this study, Basic Local Alignment Search Tool (BLAST) was used for annotation with Blast2GO software (Conesa et al., 2005;

https://www.blast2go.com/b2ghome).

Molecular cloning

In order to construct expression vectors containing corticosteroid receptors (CRs), mRNA sequences encoding catshark GR and MR were determined by 5' and 3'RACE methods with cDNA of adult liver, as described in chapter 2-2. Full-length cDNAs for coding regions of catshark and elephant fish CRs were then amplified by RT-PCR using high-fidelity KOD -Plus- (TOYOBO, Japan) and gene-specific primer sets as follows: cmGR-1Fw, GTC TTT TGC AGG TGG TGG TT (Sense); cmGR-1Re, CCG TCA CTT CTG GTG AAA CC (Antisense); cmMR-1Fw, TGC GGA AGA ACT GTG GA (Sense); cmMR-1Re, CTC ACC TGT TTT GCT GGC TT (Antisense); stGR-1Fw, GCA GAT TGT GCT GTG AGT GC (Sense); stGR-1Re, GGC AGT CAT TTG TGG TGA AA (Antisense); stMR-1Fw, GGT GTA TTA AAG AAA CAG CAA TGG (Sense); stMR-1Re, AAG GAG GAA AAT TTA AGG CAA AG (Antisense). All ampricons were subcloned into pcDNA3.1 Hygro(-) expression vector (Life technologies), and the inserted sequences were confirmed by an automated DNA sequencer (PRISM 3130, Life technologies). Gene-specific primers of elephant fish were designed based on putative coding sequences found in the Elephant Shark Genome Database (http://esharkgenome.imcb.a-star.edu.sg/).

Quantitative PCR analysis

Tissue distribution of GR and MR in adult fish and effects of steroid administration on OUC enzyme mRNAs were examined by real-time quantitative PCR (qPCR) as described in chapter 2-1. For adult fish, cDNA prepared from the brain, hypothalamus and pituitary, heart, intestine, spleen, liver, muscle, kidney, and rectal gland were used as templates. The primer sets used in qPCR analyses were

as follows: cmGR-2Fw, AGC AGT AGA AGG ACA GCA TAA C (Sense); cmGR-2Re, GGC AGG AGT CAG TAA TGA AGA A (Antisense); cmMR-2Fw, GCT GGC AGA GAA ACA GAT GA (Sense); cmMR-2Re, CCA AGA GTA ACG GAG AAG AAC C (Antisense); stGR-2Fw, ACG TTG CCA GTT TTG ACT TGA G (Sense); stGR-2Re, TGA AAT CCG GAC CTC CTA CAT T (Antisense); stMR-2Fw, CAC GGA AGT CTA AGA AGA TGG G (Sense); stMR-2Re, TGT GTC TGC TTG ACT GCT ATC (Antisense).

Molecular Phylogenetic Analysis

The deduced amino acid sequences of elephant fish and catshark CRs were aligned with those of other vertebrate CRs using ClustalX software (http://www.clustal.org/). The 21 sequences of known vertebrate CRs were included in the analysis. The molecular phylogenetic tree was generated by a Maximum likelihood method (Whelan and Goldman, 2001) with Molecular Evolutionary Genetics Analysis software (Tamura et al., 2013; MEGA version 6, http://www.megasoftware.net/). The reliability of the generated tree was shown by bootstrap values, obtained by 2,000 resampling with WAG model.

Reporter gene assay

Reporter gene assay of cloned CRs was conducted using Chinese hamster ovary (CHO) dhfr (-) cells transiently expressing CRs and secreted alkaline phosphatase (SEAP) method. The CHO cells were seeded at a density of 1 x 10^5 cells/well in 24-well plates in MEM-alpha (Life technologies), containing 10% fetal bovine serum (Hyclone, South Logan, UT, USA) and 1 x penicillin/streptomycin (Life techonologies) and cultured at 37 ^oC for 24 hours in a CO_2 incubator. Subsequently, the cells in each well were transfected with 0.6 μg pGRE-SEAP

(TaKaRa), 0.1 μg pCMV-LacZ (TaKaRa) and 0.1 μg the pcDNA3.1 expression vectors containing the cloned CRs, using 1 μL Lipofectamine 2000 in 400 μL OptiMEM (Life technologies). For positive control, the pcDNA3.1 expression vector, in which Japanese medaka (*Oryzias latipes*) olGR2 was inserted, was used (kindly gifted by Dr. S. Miyagawa and Prof. T. Iguchi of National Institute for Basic Biology). Within 12 hours, culture medium was replaced by 400 μL MEM-alpha, and ligands at final concentration of 1 x 10^{-6} M to 1 x 10^{-13} M or control vehicle were applied to the medium in duplicate. The medium was sampled after 24 hours, and the SEAP activities were measured with SEAP Reporter Gene Assay, according to the manufacturer's protocol (Roche). The chemiluminescent signals of each well were exposed to X-ray film, and the signal intensity was semi-quantitatively calculated by ImageJ (Abràmoff et al., 2004), and then normalized by transfection efficiency with β-Galactosidase Enzyme Assay System (Promega). Each value in the Figures 29 to 31 was represented by ratio to the vehicle (ethanol) control. The concentration of vehicle ethanol in culturing medium did not exceed 0.1%.

***In vivo* administration experiments**

After opening the egg capsule, embryo with yolk at stage 32 was taken out from the capsule, and was cultivated in a 200 mL plastic container with 150 mL aerated SW. In this experiment, three experimental groups were designed as follows: ethanol control (Group 1); administration of corticosterone (B) at final concentration of 1 x 10^{-6} M (Group 2); co-administration of B and RU486 at final concentration of 1 x 10^{-6} M (Group 3). The embryos were kept in SW for 3 hours, and then ethanol or ligands were added to each container. After 72 hours, embryo, ectodermal and mesoendodermal layers of YSM were dissected out and were flash frozen in liquid nitrogen. Total RNA was extracted from the frozen samples with

ISOGEN (Nippon Gene), and cDNA was synthesized as described in chapter 2-1 for qPCR analysis. Seawater was also collected from each container every 24 hours for measurement of urea concentration. The concentration of vehicle EtOH in culturing medium did not exceed 0.1%.

Statistical analysis

Data are represented as means ± SEM. Steel's non-parametric rank sum test was used for analysis of the data of the mRNA expressions between the embryo and two cell layers of the YSM. Dunnett's multiple comparison tests were conducted for *in vivo* administration experiment. *P* values less than 0.05 were considered statistically significant.

RESULTS

RNA-seq analysis

I obtained 2,878 and 2,671 assembled contigs, and 57,485 and 65,895 unassembled singletons from the RNA-Seq analyses for catshark YSM and embryo, respectively (Table 10). Since a gene database does not exist for catshark, the tBLASTx search was conducted for annotation of the obtained sequences using the public database. In the case of YSM, candidate gene names were suggested for 2,551 contigs and 27,671 singletons. I found transcription factors and hormone receptors in the annotated contigs and singletons as follows: 17 reads for nuclear receptors, 46 for G protein-coupled receptors, 201 for LDL receptors and 12 for other receptors (Table 11). In the present study, I focused on GR and MR, and the full-length coding sequences were cloned for subsequent analyses.

Cloning of cartilaginous fish GRs and MRs

The cDNA sequences encoding catshark (st) GR and MR were determined by 5' and 3' RACE methods using gene specific primers designed based on the partial sequences of putative stGR and stMR from the transcriptome analysis. For elephant fish (cm) GR and MR, candidate sequences were found from the elephant shark genome database. Then, the full-length coding regions of stGR, stMR, cmGR and cmMR were amplified by PCR. The stGR, cmGR, stMR and cmMR mRNAs encoded putative open reading flame of 715, 770, 984 and 956 amino acid residues, respectively (Figs 23 and 24). The deduced amino acid sequences of catshark and elephant fish GRs and MRs were subdivided into 4 domains (A/B, C, D and E), according to the terminology provided by Krust et al. (1986). The domain C, known as the DNA-binding domain (DBD), of cartilaginous fish GRs and MRs showed high (over 89%) amino acid identity to the DBD sequences of other vertebrate CRs (Table 12). In the DBDs, the conserved "H1 helix" (KVFFKRAV) and "D-loop" (CAGRNDCIIDKI) motifs were found in all vertebrate CRs examined in this study (Figs 24 and 25). The "lever arm" motif (EGQHNY) was also conserved among vertebrate GRs (Fig. 24), whereas one amino acid substitution was found in stMR and cmMR (EGQQNY) (Fig. 25). In contrast, A/B and D domains showed low amino acid identity among vertebrate classes; in particular, A/B domains of cartilaginous fish GRs and MRs showed less than 30% amino acid identity to those of teleost and tetrapod GRs and MRs (Table 12). Meanwhile, the domain E, which is known as the ligand-binding domain (LBD), of cartilaginous fish GRs and MRs exhibited moderate levels of amino acid identity to other vertebrate GRs and MRs, but more than 80% identity were found among cartilaginous fish GRs and MRs. Moreover, serine (Ser) and leucine (Leu) residues found in the LBD of cartilaginous fish GRs and MRs (highlighted with rectangular boxes in Figs 24 and 25) were

conserved in MRs of tetrapods and CR of sea lamprey.

Figure 26 shows the molecular phylogenetic tree, in which the sea lamprey CR was used as an outgroup. The tree consists of two large clusters of GRs and MRs, and the obtained putative GRs (stGR and cmGR) and MRs (stMR and cmMR) were included in the GR and MR clusters, respectively.

Distribution of GRs and MRs in the adult tissues and the developing embryos.

The GR and MR mRNAs of elephant fish and catshark were ubiquitously distributed in all adult tissues examined. Relatively high mRNA levels of stGR were observed in the hypothalamus and pituitary, gill, spleen, and muscle, while stMR expression was high in the liver and muscle (Fig. 27A). In elephant fish, the cmMR mRNA was highly expressed in the cerebral tissues, heart, liver and muscle, while high expression of cmGR mRNA was observed only in the muscle (Fig. 27B).

In catshark embryos of stages 30 and 31, expression of both stGR and stMR mRNAs was observed in the embryonic body and the YSM. When the mRNA levels of YSM were measured separately between the mesendodermal inner layer and the ectodermal outer layer, surprisingly, the highest levels of stGR and stMR mRNAs were detected in the ectodermal layer (Fig. 28). The mRNA levels of stGR in the embryonic body and the YSM were higher than those of stMR.

Response of cartilaginous fish CRs to glucocorticoids

The cDNAs encoding stGR, stMR, cmGR were cloned into the expression vector, and transactivation activities of these receptors were examined by co-transfecting a SEAP reporter vector, which contains glucocorticoid response element (GRE) in the promoter region. First, in order to determine which steroids induce the transactivation activity of stGR, corticosterone (B), cortisol and

dexamethasone (DEX) were tested in concentrations ranged from 10^{-12} M to 10^{-6} M (Fig. 29). The middle and high doses (10^{-9} and 10^{-6} M) of B, cortisol, and DEX considerably upregulated the SEAP activity. A dose-dependent increase was observed in the cortisol treatment (Fig. 29). On the other hand, both the B and DEX treatments showed the maximum effects at 10^{-9} M and no further increase was observed at 10^{-6} M. However, the maximum activity represented by DEX was lower than that by B. Estoradiol (E2), which was used as a negative control, moderately induced the transactivation activity, but did not show dose-dependent increase in the SEAP activity; the lowest concentration (10^{-12} M) exhibited the highest activity.

I then examined the responses of stGR, cmGR, and stMR to the B and endogenous ligand, 1α-hydroxycorticosterone (1α-OHB) (Figs 30 and 31). The SEAP activities were dose-dependently increased by the treatments of B and 1α-OHB to stGR, cmGR, and stMR, but not for 1α-OHB to cmGR (Figs 30A-D and 31A-D). The activity was only slightly changed even when high concentrations of 1α-OHB (10^{-9} M to 10^{-7} M) were applied to cmGR (Fig. 31C). When effects of B and 1α-OHB were compared, B was more efficient for the transactivation activities of stGR and cmGR than 1α-OHB. In contrast, the 1α-OHB rather than B efficiently induced the transactivation activity of stMR (Figs 30D and 31D). The Japanese medaka (*O. latipes*) olGR2 was used as a positive control, and transfection of olGR2 resulted in higher but similar pattern of increase in SEAP activities by B and 1α-OHB, when compared with the results of stGR (Figs 30E and 31E). Co-treatments with a synthetic steroid, the mifepristone (RU486; antiglucocorticoid and antiprogesterone compound) antagonized transactivation activities of all receptors induced by B and 1α-OHB (Figs 30 and 31).

Effects of corticosterone administration on OUC enzyme mRNAs in catshark embryos

Since B caused the strongest effects in the *in vitro* reporter assay, the effects of B administration on mRNA expression were examined in developing catshark embryos. The qPCR analysis was performed on the mRNAs of catshark OUC enzymes, as well as stGR, stMR and β-Actin (stACTB) mRNAs in embryos of stage 32. Since the stACTB mRNA levels in the embryonic body were significantly decreased by the B treatment (Fig. 32I), mRNA levels of enzymes and receptors were presented as nmol/gRNA in Figure 32. Significant changes in mRNA levels were observed for the stGS, stARG and stGR. The stGS mRNA levels were significantly increased in the embryonic body and the ectodermal outer layer of the YSM, while the levels were decreased in the mesendodermal inner layer by the B treatment (Fig. 32A). The increase in stGS mRNA levels in the ectodermal layer was restored by the co-administration with the RU486, but the levels in the mesendodermal layer were further decreased by the co-administration. Meanwhile, the expression of stARG mRNA in the mesendodermal layer was significantly upregulated in the presence of B, and was restored by the co-treatment with RU486 (Fig. 32D). Interestingly, the stGR mRNA levels in the ectodermal layer were downregulated by the steroid treatment, and no further effect was observed by the co-administration with RU486. No significant changes were found in the mRNA levels of other five genes (Figs 32B, C, E, F and H).

The concentration of urea in the culturing SW was at an undetectable level in all groups at any time point.

DISCUSSION

The shift of the urea-producing organ during the development of oviparous cartilaginous fish raises a question concerning the mechanisms upregulating and downregulating urea production in the embryonic body and the YSM. Endocrine control is the most likely candidate, and indeed I found a large number of transcription factor and receptor mRNAs expressing in the YSM and the embryonic body by RNA-seq analyses. Among these, I focused on glucocorticoid and mineralocorticoid receptors (GR and MR) as candidates for upstream regulators of urea production in catshark embryos. Full length cDNAs encoding GRs and MRs were obtained from catshark and elephant fish, and I found that corticosterone, as well as plausible endogenous corticosteroid 1α-OHB, induced transactivation activities of GRs and stMR by the *in vitro* reporter assay. Finally, effects of corticosterone administration were investigated in the *in vivo* culture of catshark embryos. Administration of corticosterone altered expression of GS and ARG mRNAs, and mRNA of stGR itself.

Upregulation of OUC enzyme activities by glucocorticoids was demonstrated in fetal rat liver almost 40 years ago (Gautier et al., 1977). Recent mammalian studies have revealed that the genes of several OUC enzymes, including rate-limiting CPSI and ARG, possess glucocorticoid responsive elements (GREs) in their promoter regions (Takiguchi et al., 1988; Christoffels et al., 1998; Hazra et al., 2008). Since GR is also present in fetal rat liver (Giannopoulos, 1975), the increase in OUC enzyme activities is most likely due to the increments of OUC enzyme gene expression induced by glucocorticoids via the GR. Consistent with the mammalian data, the mRNA levels of stARG were increased by corticosterone treatment, while the increase was reversed by co-administration with RU486, an antiglucocorticoid

compound, in the mesendodermal layer of the YSM, in which all OUC enzymes are highly expressed. Since ARG catalyzes the final step of urea synthesis in the OUC, it is probable that the upregulation in stARG mRNA levels directly affects the urea production rate in developing catshark embryos.

The corticosterone treatment also induced changes in stGS mRNA levels. Interestingly, the patterns of changes varied among target tissues. In the embryonic body and the ectodermal layer of YSM, the corticosterone treatment upregulated stGS mRNA levels; the increase was reversed by co-administration with RU486 in the ectodermal layer of YSM. On the other hand, the levels of stGS mRNA in the mesendodermal layer of YSM were downregulated by the corticosterone treatment. At this moment, the reason for the discrepancy in responses to corticosterone treatment is unknown. During the late phase of development, expression of OUC enzyme mRNAs was decreased in the mesendodermal layer of YSM, while that in the embryonic body was increased, resulting in a shift of the urea-producing site from the YSM to the embryonic liver. In this viewpoint, the observed opposite responses of stGS mRNA expression between the YSM and the embryonic body are of great interest. Further analysis on corticosteroid levels in embryos is necessary to determine whether the observed effects on OUC enzyme mRNAs really do happen in intact embryos. In addition, an existence of GRE in the promoter regions of OUC enzyme genes must be clarified to demonstrate direct action of GR and/or MR on OUC enzyme gene expression.

In the present study, expression of stGR and stMR mRNAs was found in both embryonic body and YSM of catshark embryos. However, surprisingly, the highest expression of stGR and stMR mRNAs was detected in the ectodermal outer layer of the YSM, where expression of OUC enzyme mRNAs was hardly detectable. It is possible that stGR and stMR contribute to other functions in the outer layer

rather than the regulation of OUC mRNA expression. In teleosts and mammals, it has been demonstrated that epithelial transports through transcellular and paracellular pathways are regulated by corticosteroids in an *in vitro* culturing system (Zettl et al., 1992; Weidenfeller et al., 2005; Kelly and Chasiotis, 2011). Moreover, upstream promoter sequences of genes encoding human tight junction proteins, occludin and claudin-5, contain functional enhancer elements responsive to glucocorticoids (Felinski et al., 2008). Since the ectodermal outer layer of YSM forms the most outer boundary separating internal and external environments, the GR and MR found in the ectodermal layer may control epithelial permeability to maintain internal homeostasis. In the ectodermal layer of YSM, the corticosterone treatment downregulated stGR mRNA levels, implying a potential auto-downregulation of stGR gene expression, as has been observed in mammals and teleosts (Rosewicz et al., 1988; Burnstein et al., 1991; Sathiyaa and Vijayan, 2003).

The cloning and molecular phylogenetic analyses revealed that cartilaginous fish have both GR and MR, as has been demonstrated in bony fish and tetrapods (Thornton, 2001; Carroll et al., 2011). Since only one corticoid receptor has been found in sea lamprey, the diversification of corticoid receptor family most probably occurred in the early gnathostomes. The GRs and MRs of elephant fish and catshark share domain structures with other vertebrate corticosteroid receptors: highly variable N-terminal region (A/B domain), DNA-binding domain (DBD), hinge domain, and ligand-binding domain (LBD) (Kumar and Thompson, 1999; Klinge, 2000; Oka et al., 2013). The DBD showed extremely high similarity among all vertebrate corticosteroid receptors. In particular, N-terminal "H1 helix" (KVFFKRAV), "lever arm" (EGQHNY), and "D-loop" (CAGRNDCIIDKI) were conserved among all vertebrate GRs and sea lamprey CR, whereas C-terminal "H2 helix" varied. It is considered that the H1 helix makes contacts with target DNA,

while H2 overlays H1 and contributes to the stabilization of the protein-DNA complex (see Bain et al., 2007). The lever arm is suggested to be important for modulation of gene-specific events subsequent to GR-DNA binding (Meijsing et al., 2009). Therefore, the high sequence identities of the H1 and lever arm of cartilaginous fish GRs indicate that the regulatory mechanisms of target gene transcription are also conserved in cartilaginous fish GRs.

Unlike the DBDs, sequence identities of ligand-binding domains (LBDs) were not extremely high. The high (over 80%) homology was observed only between cartilaginous fish GRs and between cartilaginous fish MRs; homology between stGR and stMR was only 67%, implying that the LBD has diversified. Bridgham et al. (2006) and Baker et al. (2013) investigated ligand selectivity using the LBDs of various vertebrate corticoid receptors. They suggested that two amino acid residues (Ser and Leu) in the LBD of tetrapod MRs are essential for the sensitivity to aldosterone, and these two amino acids are conserved in lamprey CR and skate GR and MR. The LBDs of lamprey and skate CRs indeed exhibited high responsiveness to aldosterone. Since the LBDs of elephant fish and catshark CRs also contain Ser and Leu residues at the corresponding sites, it is of interest to know whether cartilaginous fish GRs and MRs respond to aldosterone.

In tetrapods, GR and MR mediate function of distinct ligands, glucocorticoid (cortisol or corticosterone) and mineralocorticoid (aldosterone), respectively (Stolte et al., 2006). In teleosts, it has long been considered that cortisol performs both glucocorticoid and mineralcorticoid actions via GR and MR, respectively, but recently 11-deoxycorticosterone has been identified as a possible specific mineralcorticoid (Sturm et al., 2005; Takahashi and Sakamoto, 2013). Dissimilar to tetrapods and teleosts, only one unique corticosteroid 1α–OHB has been identified as a principal circulating corticosteroid in cartilaginous fishes (Idler

and Truscott, 1966; Truscott and Idler, 1968; Hazon and Henderson, 1984). In the present study, ligand selectivities of receptors were examined by using constructs carrying full-length coding sequences of cartilaginous fish GRs and MRs. To the best of my knowledge, this is the first report to investigate the transactivation activity using the full-length receptors in cartilaginous fish. I found that corticosterone was more effective with elephant fish and catshark GRs compared to 1α-OHB. My results are consistent with the preceding studies, in which fusion constructs of the GAL4 DBD with LBDs of putative cartilaginous fish CRs were used. They showed 10 to 100 times higher transactivation activity by corticosterone than by 1α-OHB (Bridgham et al., 2006; Carroll et al., 2008; Carroll et al., 2011). The presence of corticosterone in plasma was demonstrated in several species of cartilaginous fishes, although the plasma concentration of corticosterone is reported as less than 10% that of 1α-OHB (Truscott and Idler, 1972). Given the high effectiveness of corticosterone to GRs, corticosterone may act as endogenous glucocorticoid via GR in cartilaginous fish. In contrast, the transactivation activity of stMR was increased with both corticosterone and 1α-OHB in a similar manner, with slightly higher sensitivity to 1α-OHB. Therefore, distinct endogenous ligands may function as glucocorticoid and mineralcorticoid via GR and MR, respectively, also in cartilaginous fish. More precise analyses on receptor transactivation using multiple endogenous and exogenous ligands and on circulating corticosteroid levels are required to clarify this possibility.

Table 10. Read and alignment information of 454 sequencing

Sample	Number of reads	Total bases	Number of assembled contigs	Number of singleton	Average read length in bases (Mean ± S.D.)	RIN (RNA integrity number) of total RNA
Catshark embryo	143,854	62,768,874	2,671	65,895	436.3 ± 124.5	8.9
Catshark YSM	156,783	66,102,634	2,878	57,485	421.6 ± 117.2	9.9

Table 11. Predicted transcription factors and hormone receptors in the YSM

Protein family and annotated gene names	Read numbers
Nuclear receptor family	
COUP transcription factor 2 (NR2F2, COUP-TFII)	1
Glucocorticoid receptor (NR3C1,GR)	3
Liver X receptor α (NR1H3, LXRA)	1
Mineralocorticoid receptor (NR3C2, MR)	1
Retinoic acid receptor α (NR1B1, RAR-α)	2
Retinoic acid receptor β (NR1B2, RAR-β)	2
Retinoid X receptor α (NR2B1, RXRA)	1
Retinoid X receptor β (NR2B2, RXRB)	1
Other orphan receptors	5
G protein-coupled receptor (GPCR) family	
Endothelin receptor (ET)	4
Glucagon receptor (GCGR)	1
Neuropeptide Y receptor Y8	2
Prostaglandin E2 receptor (EP_2)	2
Other uncharacterized or orphan receptors	37
LDL receptor family	
	201
Receptors of cytokines	
	159
Other receptors	
Adiponectin receptor 1 (ADIPOR1)	6
Insulin-like growth factor 2 receptor (IGF2R)	2
Natriuretic peptide receptor (NPR-B)	3
Renin receptor (ATP6AP2)	1

(A) Catshark GR

```
  1: MANFAEDEKMKPGKQIWNVVAGLSEGSGSLKDCEGVMEGGEMMTAMPGPIMQFLPHDSNN
 61: SNKQQLDGVRIANGASGQQQHLSQAASDLSELEQSLAALNELQDAGNRVKSDTDVGLYQS
121: GCEDDFLGVLEDGSSVDFLPDLEFFPSPREEKQQGSPWSLTAFCGDDELGLLSPLSSEPP
181: LLKDSLVDLQPIEECIAPWPTLESRPTESRENIWTPLLAATTAPKEVKQEQESYIELVTP
241: GVIKQEQVSRGYCRAANGAAGAHIAGKESIYLYSTEPVQTSSDQKPTFSFVSPFTTVAQA
301: WEIRQRPNANLSESRMGNICSGRGVLPAKYNSTDLKSEGSLSTASTSTAASGPPAKVCLV
361: CSDEASGCHYGVLTCGSCKVFFKRAVEGQHNYLCAGRNDCIIDKIRRKNCPACRYRKCLQ
421: AGMNLDARKTKKLNKMKAPHTTTSETAAKTPSPDSSLVCSPLPTLNLPMISILELIEPDL
481: LYAGYDSTLPDTPNRLLSGLNNLGGLQMVSVVKWAKTLPGFRSLHLDDQMILLQYSWMSL
541: MVFSLGWRSYQHVNSSMLFFAPDLVFNEERMQQSSMYSLCKGMSNIAMDFRNLKVAYEEY
601: LCMKALLLLGTIPIDSLKSQAMFDEIRTSYIKELGKAIVKKEGSSSQNWQRFYQLTKLLD
661: SMHDLVEGLLQFCFYTFVESKTLSVEFPEMLVEIISNQLPKVMAGMAKQLRFHHK*  715
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(B) Elephant fish GR

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  1: MASFTEDERLKSKEQVWVETSDGLIQGRSDLKESQGLTGGGMMLTVASSSLLHHLPPDNR
 61: NTSDGVDITNGGGLQQNELNRTAASNLADLERSLASLSELQESVSGVESGLYLGCQTGSG
121: DSLLGLEDGISVDFLSDLEFLPSPRGDKHGSPWSLTAFFEEPGLLSPLLGDRVVDEEPLE
181: GKWDTNCTIVAEASQPSLSSLTEAPRPSLSSLTEAPRPSLSSLTTVPRSSLSSLTTTPRP
241: SLSSLTTAPRPSLSSLTTAPRPSLSSLTEIPQPSLSSLTEAPRPQPPIEVKQESYIELIT
301: PGVIKQEQADRGYCRESCGAETALGREQKHTYMFCSAQAHTSSDHRPIFSVIPTFSEIAK
361: TWDKCQSSKSTATNGVKDNYPRRAALPPKYNSSVLKFDESTPSSSSSSGPPAKVCLVCSD
421: EASGCHYGVLTCGSCKVFFKRAVEGQHNYLCAGRNDCIIDKIRRKNCPACRFRKCLQAGM
481: NLDARKTKKLNKLKAPHSSSSPSTFTQPVTKSAALVSSLLTPAMISILEGIEPDVIYASY
541: DSTIPDTPHRLLSGLNTLGGRQMISAVKWAKTIPGFQNLHLDDQMMLLQYSWMSLMVFSL
601: GWRSYQYTNSNMLFFAPDLILNEDRMQQSSMYELCKGMHSISIEFRNLQVSYEEFLCMKT
661: LLLLSTVPLDGLKSQAAFDEIRTCFIKELGKAIVKREGNASQNWQRFYQLTKLMDSMHDL
721: VGGLLQFCFYTFMESKTLSVEFPEMLVEIISNQLPKVMAGMAKPLRFHQK*  770
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Figure 24. The deduced amino acid sequences of (A) catshark and (B) elephant fish glucocorticoid receptor (GR). Numbers on the left side indicate the number of amino acid residues. The DNA-binding domains (DBD) are shown in red. In the DBDs, the sequences of H1 helix, lever arm, and D-loop are highlighted with rectangular box, black background, and blue background, respectively. Ligand-binding domains are marked with gray background. The conserved Ser and Leu residues are highlighted with rectangular boxes.

(A) Catshark MR

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  1: METKGYIDYPEVMNIGRQWSLNKSTSNVMRDEIGGCDSNAMAILNVSCIPPVAKNRDGER
 61: SQEFSPILQHQPPYHVQHQDQHLGFLASDPKCSLGAALELSKTVAESMGLYMNSAKEMGS
121: EFNYSMQPEQMEGRSSNITEIGGQEVNLQEEKVNAIISHRGMRRCSSSSSPGRNSHGSCI
181: SSPVNFNNLGCSVSSPNNINSCVPSPAKINYLALSSPDTVSNMRSSISSPDNNLSTSATS
241: PSDIHCAISSPTKINSLGMSSPNNMNSLGSSISSPANISNLHSSVSSPNNMSSPLSTPSN
301: LASPLSVSANNNILSSPNNNGMASSVSSPAVNNLRFSNSSPAAETRMIVNPHQNPRARIQ
361: KQVEIKVFKDEKIKSECLETGSINNVNPVQFMKTEVDSDFGGPCFQSRGSSDPSNVMFST
421: PLKCEMNETACLNAMYTAQVSENPFSEIAHQLIEAKTSYGDTFDLYGILGIPTSSSNSSY
481: EHDAYLQPNPTTVIKQEPNDEGYCQATCSPPSTIVGVNSTGQSFHYRIGASGTISVPCPF
541: MRDQRNQLLNLITPVCTVLGSWKSHPITPQSSISPGRSDGYPLQGYIPEKMARSPIRLDR
601: SCSSVTSAPSKVCLVCADEASGCHYGVLTCGSCKVFFKRAVEGQQNYLCAGRNDCIIDKI
661: RRKNCPACRLRKCFKAGMNLGARKSKKMGKVKGLHEEQQPPHSPKEGITFTAPLPEPTAS
721: TALVPNFSLMPPHINPSLITILEAIEPDVVYASYDSSQADTTNHLLSSLNKLAEKQMVRI
781: VKWAKGLPGFRTMALDDQMTLLRYSWMCLMTFGLSWRSYKHTSGTMLYFAPDLVFNEQRM
841: QQSAMYELCQGMQSISIEFVRLQLTYEEFLCMKAILLLGTIPKDGLKSQASFDEMRTSYI
901: KELRRVIARNENNSGQNWQRFYQLTKVLDCMHELVSGLLQFCFYTFVESKALKIEFPDML
961: VEIINDQLPEVTAGMTKSLLFHKK*  984
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(B) Elephant fish MR

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  1: MGDEIGDYSGLTVLNISYIPPVTKLHAGEESQRFFTHLQHHHSHQQCRHQHVCLISDPKC
 61: NGGSAKELSKTVAESMGLYMNSVKEMGNEFNYGLHSEQMDGRPGNNSESDGQEISLQSEE
121: KMNSILLKQGKRRCSSSSSPGRSSLGSCMSSPTSFNNLGSAMSSPNNMGSCVSSPSKINC
181: LALSSPDTVSNLGSSIASPDNINNLSTSVASPSAVRCSISSPSKINNLTMTSPSNMNSLG
241: SSISNPANVNNLRSSVSSPNNLSSPTSIPTNLASPSSAPVDNNMLGSSLYSPSNNNRVGS
301: SAYRPVLNNLHFPPLNPSNGLRLVLDSPQNTGARSQSNREMNVFKEEKPKVEGLETGMLN
361: NMNPVQFIKTEVDSDFSESCFLSRGSSELTDSTFSLPIKCEMNEDACLNMMYDSQVSENP
421: FSEIAHLTNDGKMSYGDTFDICGILGAAASSSTSSYEHDVFLQPILTPVIKQEPNDESYC
481: QGSCSSPSTVVGVNSTGQSFHYRIGAGGTISLPRPYMKDQRQQFVNLIPPVCTLLGSWKS
541: RPIMTQISTPPGRSDGYPVQGYIPENMSRSSIISSSVTSGPSKVCLVCSDEASGCHYGVL
601: TCGSCKVFFKRAVEGQQNYLCAGRNDCIIDKIRRKNCPACRLRKCLKAGMNLGARKSKKL
661: GKVKGVHEDQQPPHSPKEGITFTAPLPEPTASTAAIPHYSLLPMHINHSVTTVLQVIEPE
721: VVYACYDSSVPDNTTHLLSSLNRLAEKQMIRIVKWAKVLPGFRNLALDDQMVLLRYSWMS
781: LMSFGLSWRSFKHTNGTMLYFAPDLIFDEQRMQKSAMYELCQGMQRIGLEFVRLQLTYEE
841: FLCMKSILLLGTIPKDGLNSLANFEEMRANYIKELRRIIARNENNSGQNWQRFYQLTKIL
901: DCMHELAGGLLQFCFYTFMESKALNIEFPDMLVEIINDQLPEVTTGMTKSLYFHKK* 956
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Figure 25. The deduced amino acid sequences of (A) catshark and (B) elephant fish mineralocorticoid receptor (MR). Numbers on the left side indicate the number of amino acid residues. The DNA-binding domains (DBD) are shown in red. In the DBDs, the sequences of H1 helix, lever arm, and D-loop are highlighted with rectangular box, black background, and blue background, respectively. Ligand-binding domains are marked with gray background. The conserved Ser and Leu residues are highlighted with rectangular boxes.

Table 12. The amino acid identities of the four functional domains (A/B, C, D, and E) of stGR and stMR compared to other vertebrate GRs and MRs.

Catshark GR (stGR)				
	A/B	C (DBD)	D	E (LBD)
Sea lamprey CR	26	94	25	56
Elephant fish GR	35	98	51	80
Elephant fish MR	17	95	34	60
Catshark MR	24	92	31	67
Little skate GR	-	92	63	90
Zebrafish GR a	26	95	20	68
Xenopus GR	26	98	31	63
Chicken GR	26	98	36	65
Human GR	28	98	31	62

Catshark MR (stMR)				
	A/B	C (DBD)	D	E (LBD)
Sea lamprey CR	29	91	33	60
Elephant fish GR	21	92	42	66
Elephant fish MR	63	97	83	82
Little skate MR	-	95	86	92
Zebrafish MR	27	91	44	67
Xenopus MR	32	89	51	66
Chicken MR	32	91	56	66
Human MR	30	89	50	68

Note. Each value shows the amino acid sequence identity (%). Since only the C-terminal halfs were cloned for little skate GR and MR, A/B domains of little skate GR and MR could not be analyzed. Accession numbers are as follows: sea lamprey (*Petromyzon marinus*) CR, **AAK20930**; little skate (*Leucoraja erinacea*) GR, **DQ382338**; zebrafish (*Danio rerio*) GR, **NM_001020711**; *Xenopus* (*Xenopus laevis*) GR, **NM_001088062**; chicken (*Gallus gallus*) GR, **NM_001037826**; human (*Homo sapiens*) GR, **NM_000176**; little skate MR, **DQ382339**; zebrafish MR, **NM_001100403**; *Xenopus* MR, **NM_001090605**; chicken (*Gallus gallus*) GR, **NM_001159345**; human (*Homo sapiens*) GR, **NM_000901**. DBD, DNA-binding domain; LBD, ligand-binding domain.

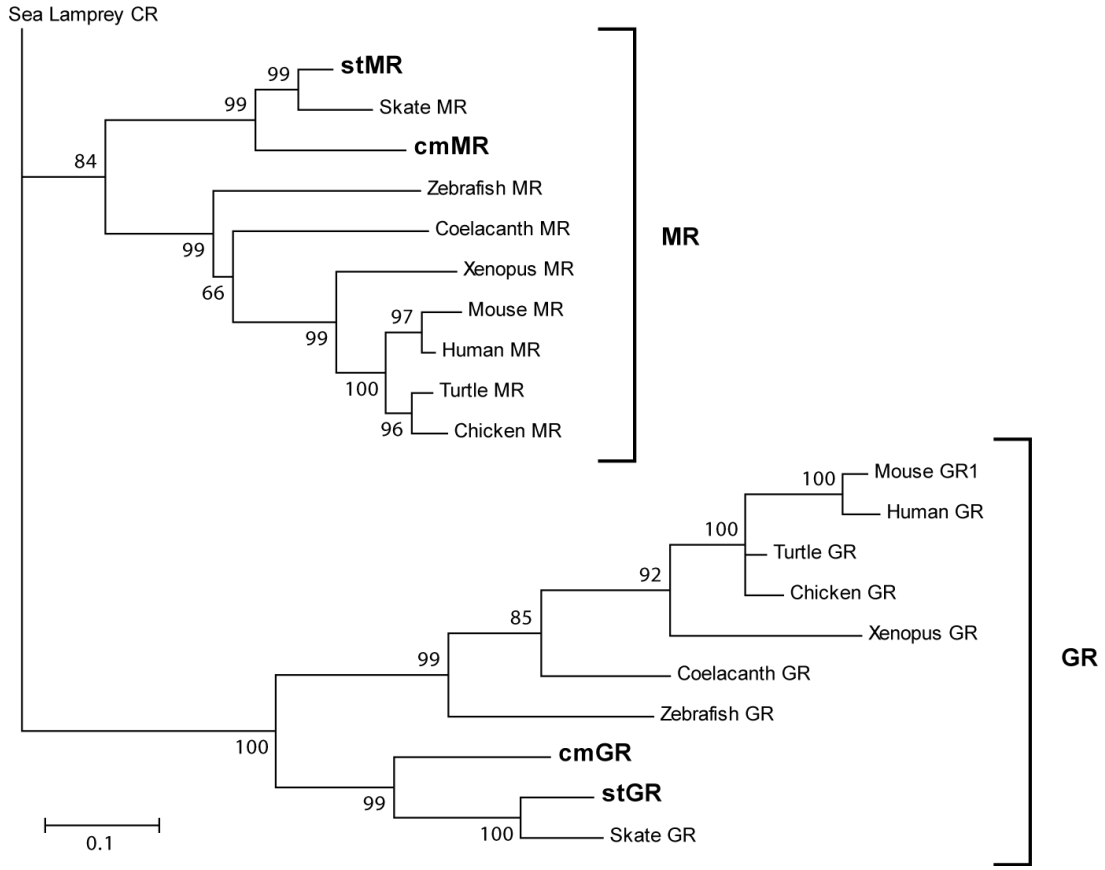

Figure 26. Molecular Phylogenetic tree of vertebrate CRs generated by the Maximum Likelihood method. The catshark CR (stGR and stMR) and elephant fish CR (cmGR and cmMR) were shown in boldface. The sea lamprey CR was used as an outgroup for the analysis. The bootstrap values are shown at the nodes of the branches. The accession numbers of predicted turtle (*Chrysemys picta bellii*) GR and MR sequences are **XM_005302851** and **XM_008166102**, respectively. The predicted sequences of coelacanth (*Latimeria chalumnae*) GR (**ENSLACG00000015535**) and MR (**ENSLACG00000010514**) were obtained from the Ensembl database.

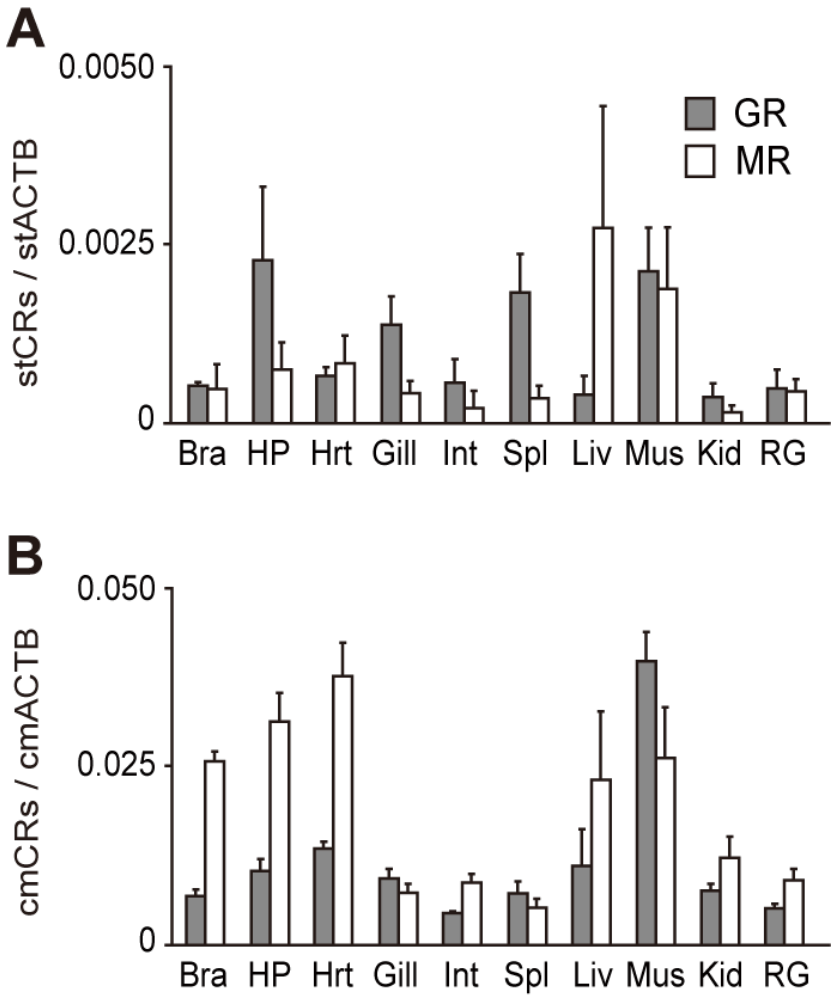

Figure 27. Tissue distribution of corticoid receptor (GR and MR) mRNAs in adult catshark (A), and elephant fish (B). The mRNA levels of CRs were normalized with ACTB mRNA levels. Bra, Brain; HP, Hypothalamus and Pituitary; Hrt, Heart; Int, Intestine; Spl, Spleen; Liv, Liver; Mus, Muscle; Kid, Kidney; RG, Rectal gland.

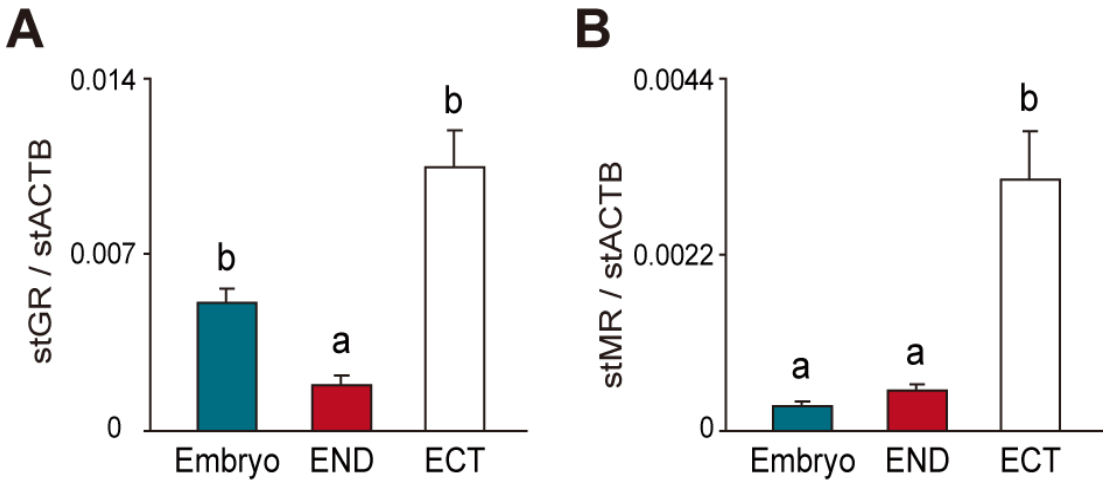

Figure 28. Distribution of catshark CR mRNAs in the embryos and extra-embryonic tissues at stages 30 and 31 (N = 12). stGR (A) and stMR (B). END, mesendodermal layer of the YSM; ECT, ectodermal layer of the YSM. All data are presented as means ± SEM (N = 6). Different letters indicate significant differences between the samples ($P < 0.05$).

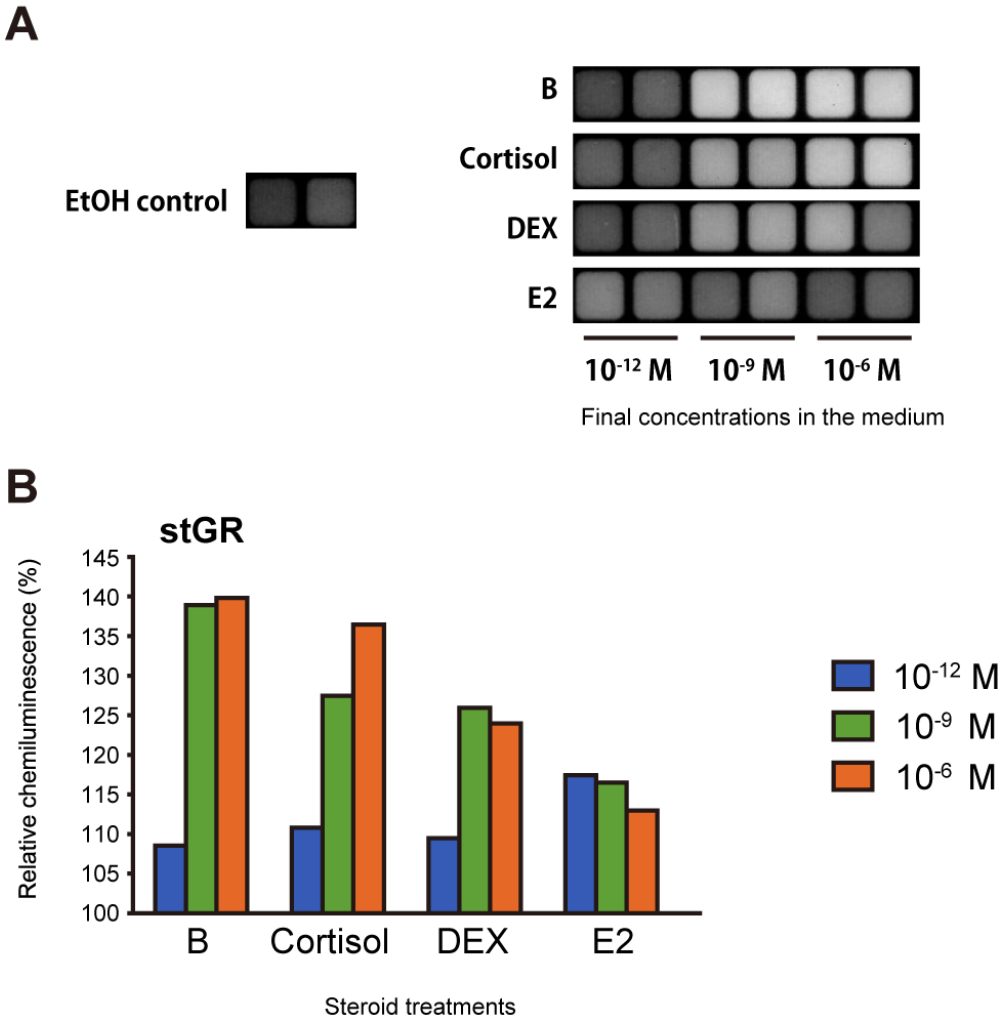

Figure 29. The transactivation activities of stGR with various ligands in the secreted alkaline phosphatase (SEAP) assay. The chemiluminescent signal of each sample was exposed to X-ray film (A), and the signal intensity was semi-quantitatively analyzed by ImageJ (B). Each value is represented as percentage to control (ethanol treatment) in (B). B, corticosterone; DEX, dexamethasone; E2, estradiol; EtOH, ethanol.

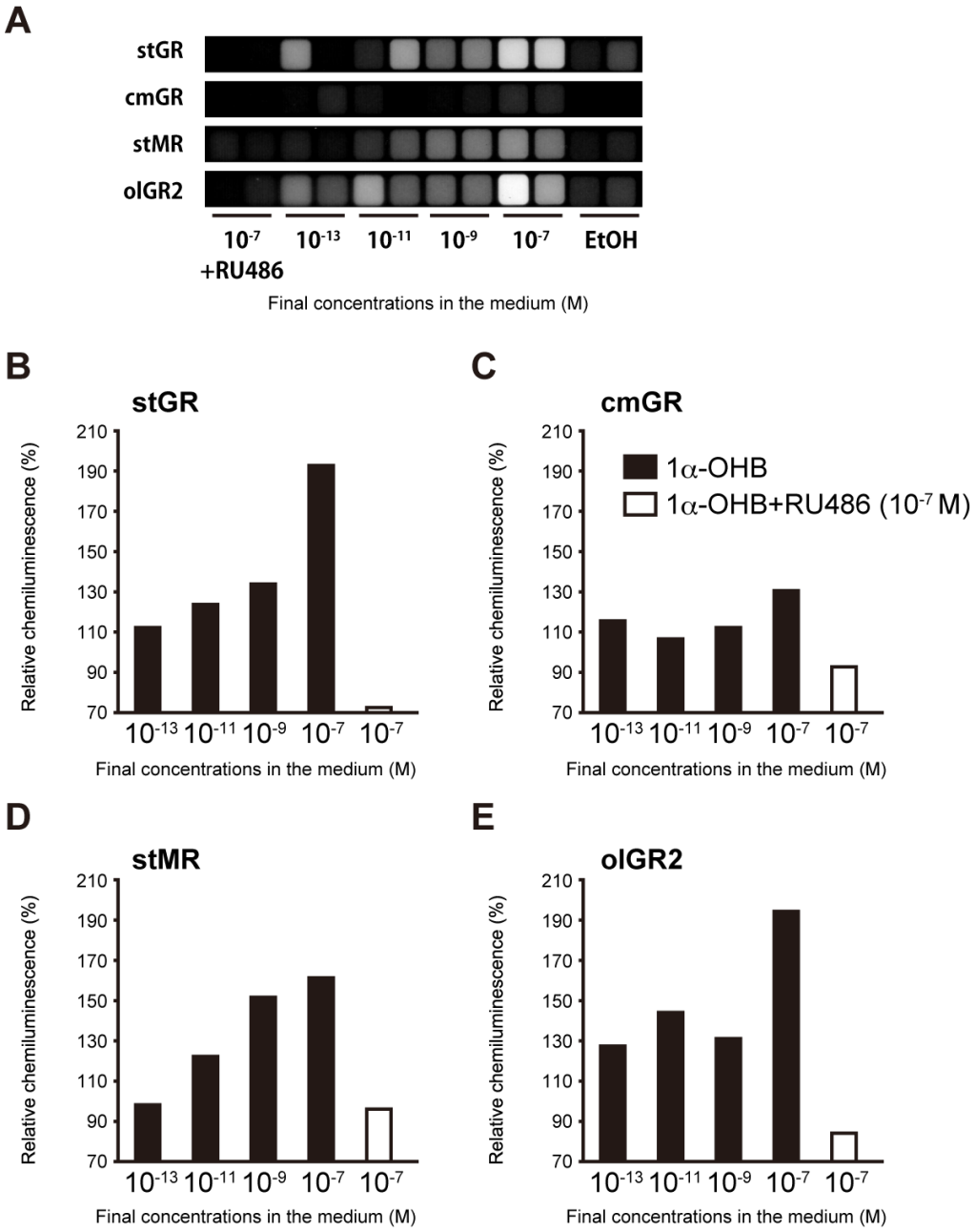

Figure 30. Effects of corticosterone (B) and B plus RU486 treatments on the transactivation activities of stGR, cmGR, stMR, and Japanese medaka (*O. latipes*) olGR2 in the SEAP assay. The chemiluminescent signal of each sample was exposed to X-ray film (A), and the signal intensity was semi-quantitatively analyzed by ImageJ (B, C, D, E). Each value is represented as percentage to control (ethanol treatment). B, corticosterone; EtOH, ethanol.

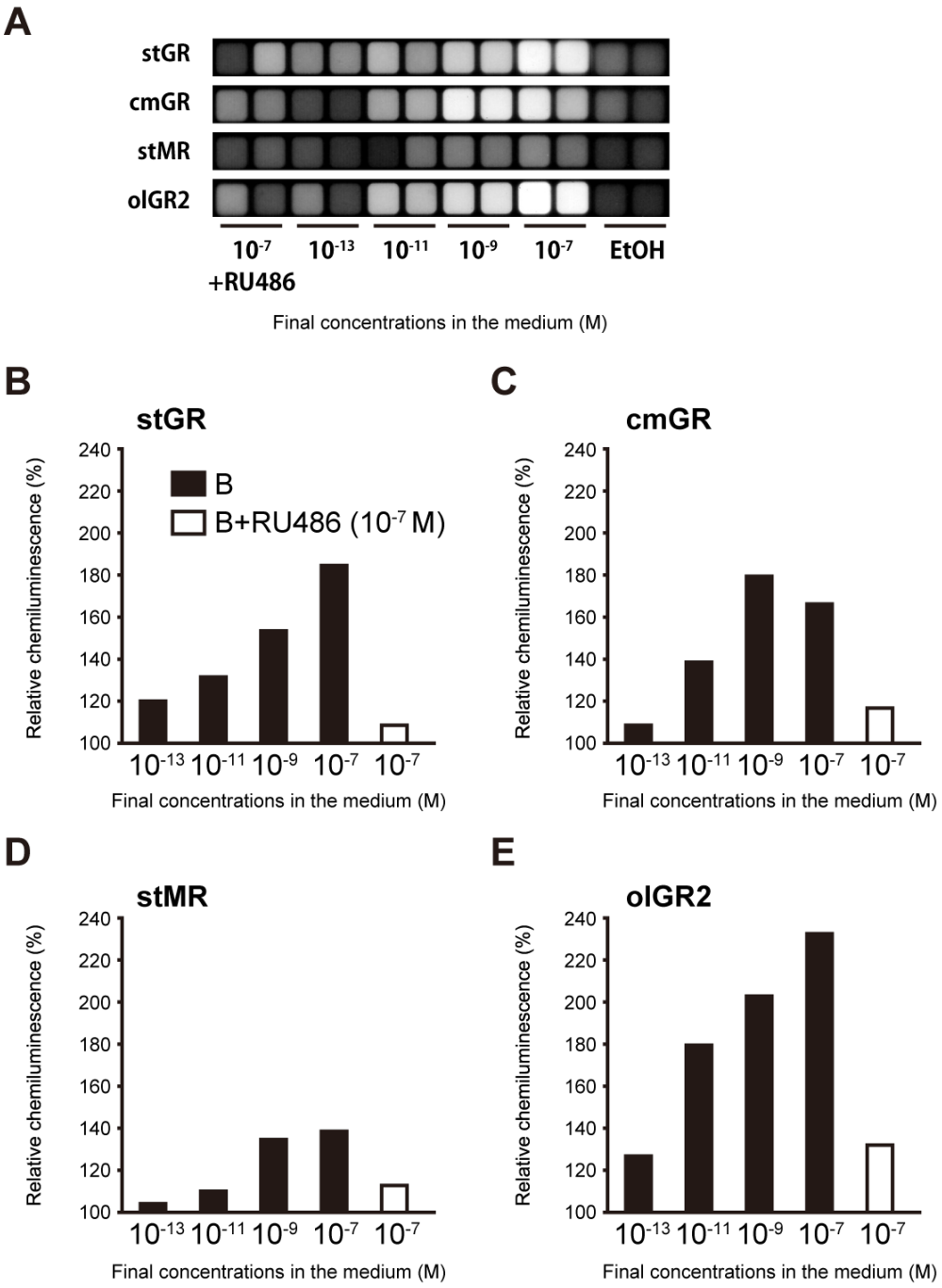

Figure 31. Effects of 1α-hydroxycorticosterone (1α-OHB) and 1α-OHB plus RU486 treatments on the transactivation activities of stGR, cmGR, stMR, and Japanese medaka (*O. latipes*) olGR2 in the SEAP assay. The chemiluminescent signal of each sample was exposed to X-ray film (A), and the signal intensity was semi-quantitatively analyzed by ImageJ (B, C, D, E). Each value is represented as percentage to control (ethanol treatment). EtOH, ethanol.

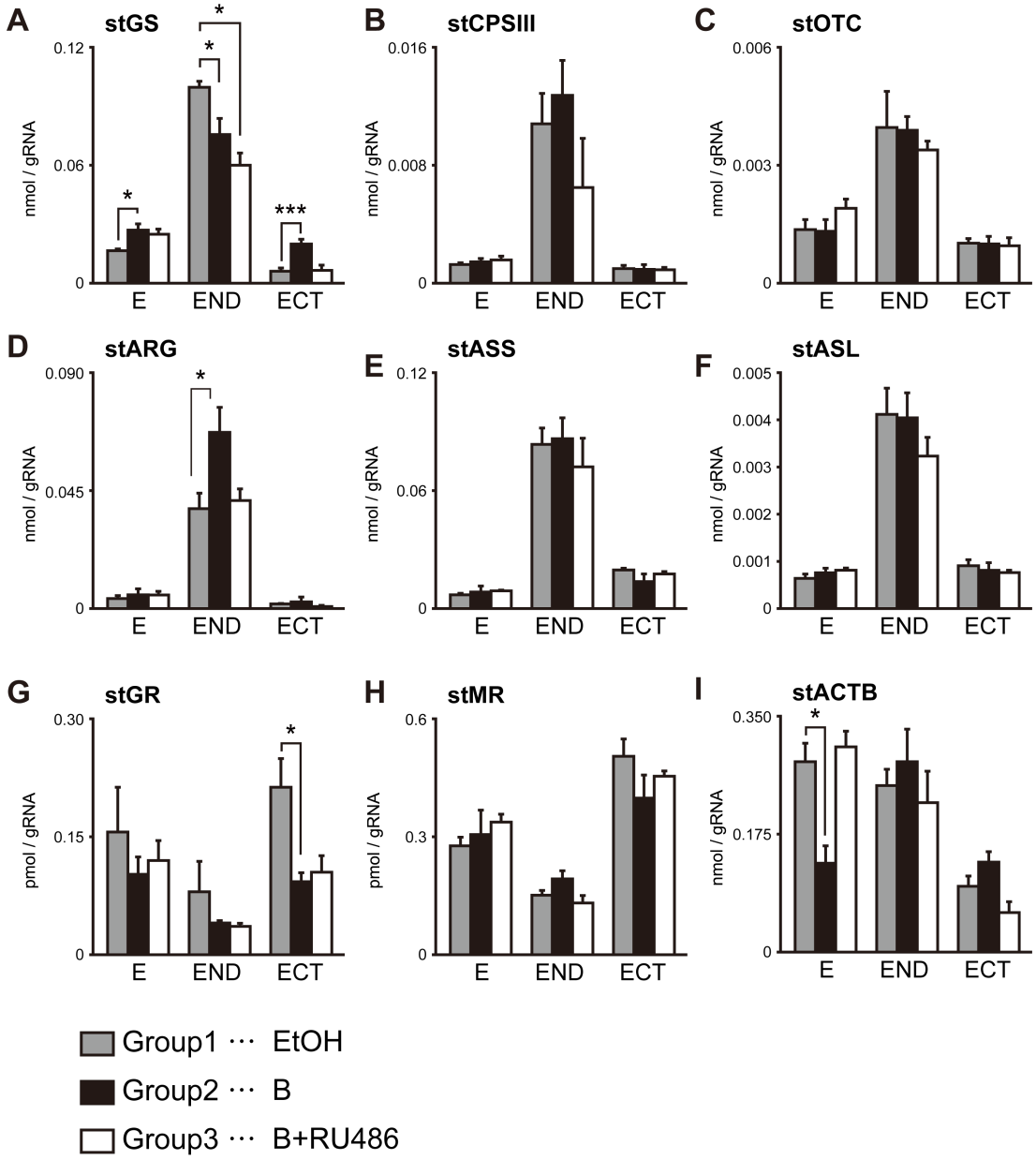

Figure 32. Changes in the mRNA expression levels of catshark OUC enzymes (A-F), CRs (G, H) and β-actin (I) after the corticosterone with or without RU486 treatments. Final concentrations of the steroids were $1x10^{-6}$ M in culturing SW. B, corticosterone; EtOH, ethanol. All data are presented as means ± SEM (N = 3). *$P < 0.05$, ***$P < 0.001$.

General Discussion

It is now recognized that strategies of aquatic animals for adaptation to a high-salinity marine environment can be classified roughly into three types. The first is the strategy called "ionic and osmotic regulator" adopted by actinopterygians. They maintain the plasma osmolality about one third that of full-strength SW regardless of their habitats, either in seawater (SW) or freshwater (FW) (Robertson, 1963; Evans, 1993; Karnaky, 1998). The contrasting strategy is "ionic and osmotic conformer", conducted by hagfish and most marine invertebrates. The third one is, needless to say, the ureosmotic strategy that has been dealt with in this thesis. Marine cartilaginous fish and coelacanth regulate their plasma ions at levels about half that of SW, while they accumulate a high concentration of urea, which corresponds to about 30-50% of plasma osmolality, to avoid osmotic dehydration caused by high-osmolality marine environment. They conform internal osmolality to the surrounding SW by actively regulating internal urea concentration. Therefore they are also osmotic conformer, and this is fundamentally different from the strategy adopted by hagfish and marine invertebrates. It is one of the central questions in vertebrate evolution of when and how the ureosmotic strategy was acquired.

The ornithine urea cycle (OUC) is a major pathway of urea production in ureotelic and ureosmotic vertebrates (Walsh and Mommsen, 2001). Although urea production has been investigated in adult elasmobranchs (sharks, skates, and rays), the osmoregulatory mechanisms of developing embryo had not been clarified yet. Thus, in the present study I investigated the urea production of marine oviparous cartilaginous fish throughout their life cycle. In the general discussion, I focus on 1) the urea production in adult cartilaginous fish, with special reference to extra-hepatic organs, 2) contribution of the yolk sac membrane (YSM) to embryonic body fluid homeostasis, and 3) the hormonal control of urea-based osmoregulation, and I will attempt to provide the future perspectives at the end of this thesis.

Urea productions in adult elephant fish: Liver is the principal urea-producing organ

In elasmobranchs, it has been well-recognized that the liver is a principal urea-producing organ. Holocephalan spotted ratfish (*Hydrolagus colliei*) also possess all the OUC enzyme activities in the liver (Read, 1967), suggesting that the major contribution of liver to urea production is a common phenomenon in cartilaginous fishes. Consistent with this idea, I found the highest relative mRNA expression and activities of carbamoyl phosphate synthetase III (cmCPSIII), ornithine transcarbamylase (cmOTC), and arginase (cmARG) in the liver of adult holocephalan elephant fish, in the present study. The predominant mRNA expression of mitochondrially-located glutamine synthetase 1 (cmGS1) was observed in the liver, supporting the presence of functional hepatic OUC. When the adult elephant fish were transferred to a low salinity environment (80% SW, 824 mOsm kg^{-1}), plasma urea levels and the resulting osmolality were reduced (Hyodo et al., 2007). Concomitantly, I found that the mRNA levels of cmCPSIII, cmOTC, cmGS1 and cmARG2 exhibited lower values than in control fish (100% SW, 1069 mOsm kg^{-1}). The downregulation in hepatic urea production and concomitant reduction in plasma urea levels have also been reported in several elasmobranch species (little skate (*Leucoraja erinacea*), lesser spotted dogfish (*Scyliorhinus canicula*) and bull shark (*Carcharhinus leucas*)), when fish were acclimated to 50% SW or freshwater (Goldstein and Forster, 1971; Hazon and Henderson, 1984; Anderson et al., 2005). These findings indicate that the decrease in plasma urea levels for adaptation to reduced environmental salinity challenges may be, at least in part, due to the reduction in hepatic urea production of elephant fish. Taken together, the liver seems to be a principal osmoregulatory organ in adult elephant fish.

Urea productions in adult elephant fish: The contributions of extra-hepatic organs

In addition to the liver, contribution of extra-hepatic organs to urea production

has become clear (Steele et al., 2005; Kajimura et al., 2006). In spiny dogfish, the activities of rate-limiting CPSIII in the muscle and intestine are greater than those in the liver, while the highest OTC and ARG activities are observed in the liver. When total skeletal muscle weight is estimated to 50% of body weight, maximum CPSIII capacity is 23-fold higher in the muscle than in the liver, suggesting that muscle may be a primary organ for urea production in this species. The mRNA expression and activities of OUC enzymes were measurable but relatively low in elephant fish muscle. However, when massive tissue mass of the skeletal muscle is similarly taken into account, the skeletal muscle appears to make a considerable contribution to urea production in elephant fish as in dogfish.

It is noteworthy that I found the high activities of OUC enzymes in the kidney of elephant fish (Chapter 1). In the kidney, the mitochondrial-type cmGS1Long was predominant, supporting the presence of functional OUC in the kidney. In marine cartilaginous fishes, the significance of the kidney for urea retention has been well demonstrated (Boylan, 1972; Hyodo et al., 2014). However, a urea-producing function of the kidney has not been documented in any cartilaginous fish species. Therefore, I investigated the localization of cmGS1 and cmOTC mRNAs in the kidney of elephant fish (Hyodo et al., 2014). *In situ* hybridization clearly showed that GS and OTC mRNAs are distributed only in limited segments of renal tubule (proximal segments of the 2nd loop), implying that the proximal segments of renal tubule contribute to urea production. Recently, a growing body of evidence has suggested that Rhesus (Rh) glycoproteins are a family of membrane proteins capable of transporting ammonia. Nakada et al. (2010) found that a novel ammonia transporter, Rhp2, is predominantly expressed in the kidney of banded houndshark (*Tryakis scyllium*) and localized in basolateral membrane of proximal segments of the 2nd loop. Therefore, I cloned mRNAs encoding Rh proteins of elephant fish, and found that

cmRhp2 mRNA was predominantly expressed in the elephant fish kidney. The signals for cmRhp2 mRNA were colocalized with cmGS1 and cmOTC mRNAs in the proximal segments of the 2nd loop. Taken together, ammonia is likely absorbed from the blood in the sinus or from the primary urine, and subsequently converted into urea via functional OUC in the limited segment of the proximal tubule. The kidney is thus important for urea production as well as its crucial function for urea retention by reabsorbing more than 90% of urea from the filtrate.

Urea-based osmoregulation in oviparous cartilaginous fish embryos: Contribution of the YSM to embryonic homeostasis

As mentioned above, the high systemic urea levels in adult cartilaginous fish are achieved by hepatic and extra-hepatic urea production. In addition to the urea-producing organs, the gill and kidney are known to be essential for urea retention. With regard to the kidney, more than 90% of urea can be reabsorbed from the filtrate (Boylan, 1972). However, developing embryos may not possess these functional osmoregulatory organs, depending on the stage of development. Therefore, my focus is whether the developing embryos of cartilaginous fish conduct urea-based osmoregulation, and if they do, how they can produce and/or retain urea without these osmoregulatory organs. Despite the long history of research on osmoregulation of marine cartilaginous fishes, urea retention mechanisms of the developing embryos have not been unraveled. This may be due to the difficulties of studying cartilaginous fish embryos. For instance, difficulty in keeping a large size of matured adult fish, sample availability and lack of staging schemes. In viviparous species, several preceding studies reported that the solute composition of uterine fluid is similar to the maternal blood during the early gestational period, and thus the embryos conceivably do not need to osmoregulate and ionoregulate by themselves (Kormanik, 1993). In the present

study, I therefore focused on oviparous species, in which the eggs are laid in SW within a day after fertilization, different from viviparous species.

In chapter 2, I found that osmolality and ion composition of the fluid inside the egg capsule are nearly identical to those of SW. Meanwhile, urea and ion composition of the embryonic body fluid are constantly maintained at levels nearly identical to those of adult fish at all developmental stages I examined. The high urea concentration was also found in the yolk throughout the development of catshark, but the concentrations in yolks were lower than those in the embryonic body (Chapter 2-2). This is probably due to the difference of water contents between yolk and embryonic body. Read (1968b) showed that water content in the embryos is 1.5 times higher than that in the yolk; the urea concentration in the embryos and yolks are nearly equal with dry mass-based correction (in the big skate *R. binoculata*, 2,354 mg urea per 100 ml water in embryos *versus* 2,420 mg urea per 100 ml water in yolks). Before the formation of YSM, the vitelline membrane, which is an extremely fragile acellular membrane, covers the yolk at oviposition and the subsequent early stages (Smith, 1936; Read, 1968b). It is therefore reasonable to consider that, during the very early stages of development, urea in yolk contributes to the maintenance of osmotic homeostasis of cells comprising embryos.

During the development, urea concentration in the yolks was maintained at a constant level. These results imply that the developing embryos do not adopt specific mechanisms for absorbing urea from the yolk for their osmoregulation. In other words, embryos must synthesize urea by themselves to maintain a high concentration of urea in the embryonic body during development. Therefore, I examined mRNA expression of OUC enzymes during the development of elephant fish and catshark. The most striking finding is the occurrence of OUC enzymes in the extra-embryonic YSM of both oviparous cartilaginous fishes. In holocephalan elephant fish, the abundance of

mRNA encoding rate-limiting cmCPSIII was notably higher in the YSM than in the whole embryonic body. The mitochondrial-type GS mRNA was also abundantly expressed in the YSM during the early developmental period (stages 28-31). The presence of enzyme activities of GS, CPSIII, OTC, ARG was also evident in the YSM of both elephant fish and catshark, implying a crucial contribution of the YSM to the embryonic urea production during early development. Furthermore, I found the shift of the urea-producing site from the YSM to the embryonic body, around the time when the yolk sac starts to be absorbed into the abdominal cavity of the embryo. Concomitant with the regression of the yolk sac, mRNA levels of OUC enzymes were decreased in the YSM but markedly increased in the embryonic body. Since the highest expression of OUC enzyme mRNAs was found in the liver of the embryo during the late developmental stages, the primary site of urea production during the late phase of development is likely the embryonic liver.

Scammon (1913, 1915) observed spiny dogfish embryonic liver in detail, and proposed that the liver development is separated into three phases: 1) hepatic diverticulum is observed as hollow buds; 2) the number of hepatic tubules increases, and roughly one-half of cross section of the liver is made up of vascular space (abundant in sinusoids); 3) the size but not number of the hepatic tubules increased, resulting in a decrease in size of the sinusoids. During the later stage of phase 3 (the embryo size is 47.3 mm), sinusoids form capillary structures. Since the complex three-dimensional architecture, comprised of hepatocyte, sinusoid, artery, portal vein, and bile duct, is critical for hepatic function (Si-Tayeb et al., 2010), it can be assumed that the liver of spiny dogfish finalizes formation and becomes functional after phase 3. In the case of catshark, I found that the liver volume of the embryo was exponentially increased from stage 23 after it apparently emerged, but the mRNA levels and activities of OUC enzymes remained low in the embryonic body until stage 32. Although

Scammon (1913, 1915) described only the size of embryos, these stages of catshark embryos most probably correspond to the phases 1 to 3 of liver development in spiny dogfish, implying that the liver of catshark is not fully functional and still in the process of formation until stage 32. Following this stage, the mRNA expression was remarkably increased, suggesting that the liver of the embryo starts to function as a primary organ for urea production after this stage. I propose to term the functional period as the "phase 4" of the liver development, but detailed morphological and functional investigations on catshark liver are necessary.

In the present study, I found the considerable contribution of the YSM to urea-based osmoregulation in oviparous cartilaginous fish embryos. When the present findings are collectively considered with recent studies in other vertebrates and invertebrates, it is obvious that the contribution of the extra-embryonic tissues to embryonic homeostasis is not limited to cartilaginous fish, and is broadly observed in other animals. Without doubt, the fundamental role of the yolk sac is absorption and digestion of yolk nutrients. A well-known representative example is chick eggs, in which the YSM transport nutrients from the yolk to the embryonic body, by expressing nutrient transporter and digestive enzyme genes (Yadgary et al., 2011). Meanwhile, the involvements of YSM in osmoregulation have been reported in teleosts (Kaneko et al., 2008). The mitochondria-rich ionocytes distributed in the YSM are responsible for ion regulation during the early developmental period of both SW and FW teleost fishes (Ayson et al., 1994; Hiroi et al., 2008). These ionocytes on the YSM respond to environmental stimuli in a similar manner to what has been observed in the ionocytes of the gills; in response to transfer from FW to SW, the absorptive-type ionocytes are replaced by the secretory-type ionocytes in the embryos of euryhaline tilapia (Hiroi et al., 1999; Katoh and Kaneko, 2003). Furthermore, similar to my findings, the distribution of ionocytes is shifted from the YSM and body surface to the embryonic

gill in teleosts, as development proceeds (Katoh et al., 2000). Recent studies also demonstrated that the YSM of some invertebrate species plays an important role in pH homeostasis, and the site of acid-base regulation is shifted to embryonic organs during development (Cieluch et al., 2005; Hu et al., 2011). Therefore, it is reasonable to hypothesize that ontogeny-dependent shifts to principal organ for body fluid regulation occurs in general in most animals, particularly in the aquatic animals.

Hormonal regulation of urea production and body fluid homeostasis in embryos

In elephant fish and catshark embryos, the mRNA expression of OUC enzymes was downregulated in the YSM, while upregulated in the embryonic body, as development proceeds. My subsequent question was whether these developmental changes are under hormonal controls. I thus performed RNA-seq analysis on the catshark YSM, and focused on corticosteroid receptors (CRs) from among a number of transcripts as candidates for regulatory factors controlling OUC enzyme mRNA levels. Since corticosterone exhibited potent transactivation activity on glucocorticoid receptor (GR), I examined effects of corticosterone on OUC enzyme mRNA levels using the *in vivo* culture system of catshark embryos, and found that the administration of corticosterone induced upregulation of stARG expression in the mesendodermal cell layer of the YSM. The upregulation was clearly reversed by co-administration with RU486, which is established GR antagonist in mammals and teleosts. This result is consistent with the preceding research on rat fetal ARG activity and mRNA expression (Lamers and Mooren, 1981; Nebes and Morris, 1988). Since ARG catalyzes the final step of urea synthesis in the OUC, it is reasonable to infer that the transcriptional regulation of ARG by corticosteroids is effective for controlling urea production, and that this regulatory mechanism has been conserved in vertebrates.

In the embryonic body of catshark, the expression of stGS mRNA was

upregulated by corticosterone treatment. It is noteworthy that stGS mRNA expression in the mesendodermal layer of YSM was downregulated by the corticosterone treatment. Mechanisms for these opposite responses in the stGS mRNA expression are not clear at this moment. Nevertheless, these opposite responses by corticosterone are of great interest from the viewpoint of opposite regulation of OUC enzyme mRNAs between the embryonic body and the mesendodermal layer of YSM during the late phase of development. In gulf toadfish (*Opsanus beta*), OUC enzyme activities were upregulated by various stresses (Walsh et al., 1994), and two glucocorticoid response elements (GREs) were found in the promoter region of the toadfish GS gene (Esbaugh and Walsh, 2009). The upregulation of stGS mRNA levels in the embryonic body is likely to be controlled directly by corticosteroids via GRE. In addition to the direct action, it should be noted that indirect regulation by corticosteroids has been observed. In mammals, corticosteroids cause changes in gene expression of transcription factors such as C/EBP-β and/or HNF-3 (Morris, 1992; Gotoh et al. 1997; Schoneveld et al. 2004), which are also strong regulators of urea cycle enzyme genes. In spiny dogfish, two putative C/EBP-β binding sites have been found in the promoter region of CPSIII gene. These multiple regulatory mechanisms by corticosteroids might cause the opposite responses in stGS gene expression. Questions also remain about the transcriptional regulation by corticosteroids on stCPSIII, stASS and stASL, whose activities or gene expression are upregulated in mammals (Hazra et al., 2008). Further investigations are necessary to clarify whether upstream promoter regions of catshark and elephant fish OUC enzyme genes contain positive and/or negative GREs.

Although several studies suggested a mineralocorticoid action of the endogenous 1α-hydroxycorticosterone (1α-OHB) in cartilaginous fish, there was no direct evidence demonstrating that 1α-OHB activates either glucocorticoid receptor (GR) or mineralocorticoid receptor (MR), or both. In chapter 3, I have cloned

full-length coding sequences of GR and MR in elephant fish and catshark, and then examined their ligand selectivities with several corticosteroids as ligands. Corticosterone treatment induced the transactivation activity of GRs more efficiently than with 1α-OHB treatment, while stMR was sensitive to both corticosterone and 1α-OHB to a similar extent, but slightly more sensitive to 1α-OHB. Although the concentration of corticosterone in the plasma of cartilaginous fishes has been reported to be less than 10% of 1α-OHB (Truscott and Idler, 1972), the results of *in vitro* transactivation assay imply that 1α-OHB cannot be simply considered to play dominant roles on GR and MR in cartilaginous fish. The low plasma levels of other corticosteroids, such as corticosterone, have the potential to be endogenous ligands for GR and MR. Based on the results of the reporter assay, it may be reasonable to consider that distinct physiological functions of corticosterone and 1α-OHB are mediated by GR and MR, respectively.

In order to test my idea, I need to investigate endogenous corticosteroid levels (1α-OHB and corticosterone) in yolk and embryo during development, as well as adult fish. I could not find the transcript of steroidogenic acute regulatory protein (StAR) in the RNA-seq data of either catshark YSM nor of embryo at stage 32, suggesting that the ligands of GR and MR are mainly derived from the maternal source in the yolk rather than *de novo* synthesis during the early to middle phase of development in the cartilaginous fish. During the development of teleost fish embryo, cortisol concentration in the whole embryo showed the highest value at oviposition, and the levels were gradually decreased towards hatching; the lowest cortisol level was observed during the hatching period (Hwang and Wu, 1993; Ayson et al., 1995). It was proposed that the cortisol production starts after hatching, and teleost embryos utilize maternal cortisol until hatching. The developmental changes in corticosteroid concentration and the development of the steroidogenic system in oviparous

cartilaginous fish embryo are the subjects of ongoing study.

Future Perspectives

The internal urea level is a balance between urea production and loss. As I already mentioned, a large concentration gradient for urea between inside and outside the embryonic system exists throughout development. In adult cartilaginous fishes, the gill, whose secondary lamellae represent a large surface area (Hughes et al., 1986), shows the lowest epithelial permeability to urea among biological membranes thus far reported, and most probably prevents passive urea efflux to a large extent (Wood et al., 1995; Pärt et al., 1998). In my thesis, I investigated the urea production in developing embryos. However, to maintain high urea levels in the body throughout development for up to 6 months, a urea-retaining mechanism must exist to cope with passive urea diffusion. Although there has been no direct evidence showing low urea permeability of the embryonic skin and the YSM at present, one possible mechanism is that the tightly sealed impermeable outer layer minimizes urea loss from the YSM, which occupies a large surface area. As I mentioned in chapter 3, the YSM of cartilaginous fish is comprised of two cellular layers. The internal mesendodermal layer covers the yolk and is highly vascularized, while the outer ectodermal layer is a very thick membrane with fibrous connective tissue. Interestingly, I found that GR and MR mRNAs were abundantly expressed in the ectodermal layer of YSM, compared to other embryonic tissues. It has been demonstrated in teleosts and mammals that transepithelial electrical resistance of epithelial cells derived from many tissues are regulated by corticosteroids (Zettl et al., 1992; Weidenfeller et al., 2005; Kelly and Chasiotis, 2011). If this mechanism also exists in cartilaginous fishes, the permeability across the ectodermal layer of YSM may be regulated by corticosteroids via GR and/or MR. Furthermore, the YSM of cartilaginous fishes can be a good

experimental model for investigating regulation of cellular junction mechanisms and relevant signaling pathways at the *ex vivo* level, because of its ease of handling for physiological experiments and of its thin membranous structure appropriate for electrophysiological experiments. I already established an experimental system of the electrophysiological study using the ectodermal layer of YSM and the Ussing chamber. I succeeded in measuring transepithelial potential and transmembrane resistance using this system. I believe that this system can provide a powerful tool for investigating the mechanisms that enable urea retention in the embryonic body.

The difference in the osmoregulatory mechanisms between oviparous and viviparous species is another interesting research topic in the near future. Although I have tried to examine urea homeostasis in the developing embryos of viviparous red stingray (*Dasyatis akajei*), the difficulty in sample availability made this impossible to examine during the course of my PhD. I found that the volume of yolk is much smaller than elephant fish, and yolk is rapidly absorbed during the early phase of development in the red stingray. After yolk absorption, the mother secretes protein- and lipid-enriched "uterine milk (histotroph)" from the trophonemata of the uterus, and the embryos absorb the milk, which allows the embryos to increase several orders of magnitude larger than the eggs in mass by the time of birth. Since the YSM of stingray embryo reduces rapidly in size, contribution of YSM to urea synthesis seems to be less important in this viviparous stingray compared to oviparous species.

Furthermore, capacity of the developing embryos for adaptation to altered environmental salinity is also an important aspect. Almost 80 years ago, Smith (1931) found the plasma urea reduction in adult elasmobranchs acclimating to the reduced environmental salinity. Although he assumed that the urea reduction is simply due to increased urinary output and reduced renal reabsorption, recent studies on several elasmobranchs have revealed the involvements of reduced urea biosynthesis under the

low-salinity environment as I have already mentioned. Conversely, the plasma urea levels increase following transfer back to full-strength SW. These findings imply that urea synthesis is controlled in response to the environmental salinity (Anderson et al., 2005). After spawning, the eggs of oviparous cartilaginous fish are anchored to seaweeds with the tendrils or covered with sand on the sea floor. Therefore, they have to stay in the same place for a notably long period (around 6 months) until hatching without maternal protection. Thus, I assume that the embryos of oviparous species are highly adaptive to environmental salinity changes more than viviparous species. Indeed, I observed that elephant fish embryos and newly hatched fish can tolerate at least 50% SW, while direct transfer to 90% SW (just 10% decrease) was lethal to adult elephant fish. Mature elephant fish spawn in autumn (March to May in Australia) in shallow bays, and migrate back to the deep sea after spawning. On the other hand, embryos develop in shallow bays for up to 6 months. The winter of southern Australia has heavy rainfall compared to the summer, and it is reported that the salinity of bay water is diluted during the winter. Therefore, the environmental characteristics of habitat coincide well with the physiological features of adult and embryos of elephant fish, at least in terms of the salinity tolerance. What are the mechanisms for the higher tolerance in the elephant fish embryos? Since my present findings indicate the regulatory role of corticosteroids in urea production, the salinity tolerance and effect of corticosteroids should be tested for the developing embryos of oviparous and viviparous species during acclimation to low- and high-salinity environments. Are the corticosteroids freshwater- and seawater-adapting hormones in cartilaginous fish as in teleosts? The prospective findings will shed a new light on the adaptive mechanisms of the cartilaginous fish embryos to the environmental changes and may provide a cue to understanding the diversified reproductive modes.

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References

Abràmoff, M.D., Magalhães, P.J., and Ram, S.J. (2004). Image processing with ImageJ. *Biophotonics international*, **11**(7), 36-43.

Anderson, P.M. (1980). Glutamine- and *N*-acetylglutamate-dependent carbamoyl phosphate synthetase in elasmobranchs. *Science,* **208**, 291-293.

Anderson, P.M. (1995). Urea cycle in fish: molecular and mitochondrial studies. In: Wood, C.M., Shuttleworthn, T.J. (Eds.), Cellular and Molecular Approaches to Fish Ionic Regulation, Vol. 14. Fish Physiology, Academic Press, San Diego, pp. 57–83.

Anderson, P.M. (2001). Urea and glutamine synthesis: environmental influences on nitrogen excretion. In: Wright, P.A., Anderson, P.M. (Eds.), Nitrogen Excretion, Vol. 20. Fish Physiology, Academic Press, San Diego, pp. 239–278.

Anderson, P.M. and Walsh, P.J. (1995). Subcellular localization and biochemical properties of the enzymes of carbamoyl phosphate and urea synthesis in the batrachoidid fishes *Opsanus beta*, *Opsanus tau* and *Porichthys notatus*. *J. Exp. Biol.,* **198**, 755-766.

Anderson, W.G., Good, J.P., Pillans, R.D., Hazon, N., and Franklin, C.E. (2005). Hepatic urea biosynthesis in the euryhaline elasmobranch *Carcharhinus leucas*. *J. Exp. Zool.,* A **303**, 917-921.

Anderson, W.G., Pillans, R.D., Hyodo, S., Tsukada, T., Good, J.P., Takei, Y., Franklin, C.E., and Hazon, N. (2006). The effects of freshwater to seawater transfer on circulating levels of angiotensin II, C-type natriuretic peptide and arginine vasotocin in the euryhaline elasmobranch, *Carcharhinus leucas*. *Gen Comp. Endocrinol.,* **147**(1), 39-46.

Armour, K.J., O'Toole, L.B., and Hazon, N. (1993). The effect of dietary protein restriction on the secretory dynamics of 1α-hydroxycorticosterone and urea in the dogfish, *Scyliorhinus canicula*: a possible role for 1α-hydroxycorticosterone in sodium retention. *J. Endocrinol.,* **138**(2), 275-282.

Ayson, F.G., Kaneko, T., Hasegawa, S., and Hirano, T. (1994). Development of mitochondrion-rich cells in the yolk-sac membrane of embryos and larvae of tilapia, *Oreochromis mossambicus*, in fresh water and seawater. *J. Exp. Zool.,* **270**, 129-135.

Ayson, F.G., Kaneko, T., Hasegawa, S., and Hirano, T. (1995). Cortisol stimulates

the size and number of mitochondrion-rich cells in the yolk-sac membrane of embryos and larvae of tilapia (*Oreochromis mossambicus*) *in vitro* and *in vivo*. *J. Exp. Zool.*, **272**(6), 419-425.

Bain, D.L., Heneghan, A.F., Connaghan-Jones, K.D., and Miura, M.T. (2007). Nuclear receptor structure: implications for function. *Annu. Rev. Physiol.*, **69**, 201-220.

Baker, M.E., Funder, J.W., and Kattoula, S.R. (2013). Evolution of hormone selectivity in glucocorticoid and mineralocorticoid receptors. *J. Steroid Biochem. Mol. Biol.,* **137**, 57-70.

Ballantyne, J.S. and Fraser, D.I. (2013). Euryhaline elasmobranchs. In: McCormick, S.D., Farrell, A. and Brauner, C. (Eds.), Euryhaline Fishes, Vol. 32. Fish Physiology, Academic Press, San Diego, pp. 125–198.

Ballard, W.W., Mellinger, J., and Lechenault H. (1993). A series of normal stages for development of *Scyliorhinus canicula*, the lesser spotted dogfish (Chondrichthyes: Scyliorhinidae). *J. Exp. Zool.,* **267,** 319-336.

Barber, M.L. and Walsh, P.J. (1993). Interactions of acid-base status and nitrogen excretion and metabolism in the ureogenic teleost *Opsanus beta*. *J. Exp. Biol*., **185**, 87-105.

Bauer, R., Plieschnig, J.A., Finkes, T., Riegler, B., Hermann, M., and Schneider, W.J. (2013). The developing chicken yolk sac acquires nutrient transport competence by an orchestrated differentiation process of its endodermal epithelial cells. *J. Biol. Chem.*, **288**(2), 1088-1098.

Bentley, P.J. (1998). Comparative Vertebrate Endocrinology. 3rd Edition, Cambridge University Press.

Boylan, J.W. (1972). A model for passive urea reabsorption in the elasmobranch kidney. *Comp. Biochem. Physiol.* A, **42**(1), 27-30.

Bridgham, J.T., Carroll, S.M., and Thornton, J.W. (2006). Evolution of hormone-receptor complexity by molecular exploitation. *Science,* **312**(5770), 97-101.

Brown, G.W. Jr, Brown, W.R., and Cohen, P.P. (1959). Comparative biochemistry of urea synthesis. II. Levels of urea cycle enzymes in metamorphosing Rana catesbeiana tadpoles. *J. Biol. Chem.,* **234**(7), 1775-1780.

Burnstein, K.L., Bellingham, D.L., Jewell, C.M., Powell-Oliver, F.E., and

Cidlowski, J.A. (1991). Autoregulation of glucocorticoid receptor gene expression. *Steroids,* **56**(2), 52-58.

Carpenter, C.C., Davis, J.O., and Ayers, C.R. (1961). Relation of renin, angiotensin II, and experimental renal hypertension to aldosterone secretion. *J. Clin. Invest.*, **40**(11), 2026.

Carroll, S.M., Bridgham, J.T., and Thornton, J.W. (2008). Evolution of hormone signaling in elasmobranchs by exploitation of promiscuous receptors. *Mol. Biol. Evol.,* **25**(12), 2643-2652.

Carroll, S.M., Ortlund, E.A., and Thornton, J.W. (2011). Mechanisms for the evolution of a derived function in the ancestral glucocorticoid receptor. *PLoS Genet.,* **7**(6), e1002117.

Cavalli, R.C., Burke, C.J., Kawamoto, S., Soprano, D.R., and Ash, D.E. (1994). Mutagenesis of rat liver arginase expressed in *Escherichia coli*: role of conserved histidines. *Biochemistry,* **33**, 10652-10657.

Chamberlin, M.E. and Ballantyne, J.S. (1992). Glutamine metabolism in elasmobranch and agnathan muscle. *J. Exp. Zool.,* **264**, 267-272.

Chipkin, S.R., Stoff, J.S., and Aronin, N. (1988). Immunohistochemical evidence for neural mediation of VIP activity in the dogfish rectal gland. *Peptides*, **9**(1), 119-124.

Christoffels, V.M., Grange, T., Kaestner, K.H., Cole, T.J., Darlington, G.J., Croniger, C.M., and Lamers, W.H. (1998). Glucocorticoid receptor, C/EBP, HNF3, and protein kinase A coordinately activate the glucocorticoid response unit of the carbamoylphosphate synthetase I gene. *Mol. Cell. Biol.*, **18**(11), 6305-6315.

Chrousos, G.P. (2009). Stress and disorders of the stress system. *Nat. Rev. Endocrinol.*, ***5***(7), 374-381.

Cieluch, U., Charmantier, G., Grousset, E., Charmantier-Daures, M., and Anger, K. (2005). Osmoregulation, immunolocalization of Na^+/K^+-ATPase, andultrastructure of branchial epithelia in the developing brown shrimp, *Crangon crangon* (Decapoda, Caridea). *Physiol. Biochem. Zool.,* **78**, 1017-1025.

Claros, M.G. and Vincens, P. (1996). Computational method to predict mitochondrially imported proteins and their targeting sequences. *Eur. J. Biochem.*, **241**, 779-786.

Cohen, D.M. (1970). How many recent fishes are there? California Academy of

Sciences.

Compagno, L.J.V. (1981). Legend versus reality - the jaws image and shark diversity. *Oceanus*, **24**(4), 5-16.

Compagno, L.J.V. (1990). Alternative life-history styles of cartilaginous fishes in time and space. *Environ. Biol. Fish.,* **28**, 33-75.

Conesa, A., Gotz, S., Garcia-Gomez, J.M., Terol, J., Talon, M., and Robles, M. (2005). Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics,* **21**, 3674–3676.

Cooper, A.J. and Plum, F. (1987). Biochemistry and physiology of brain ammonia. *Physiol. Rev*., **67**(2), 440-519.

Didier, D.A., LeClair, E.E., and Vanbuskirk, D.R. (1998). Embryonic staging and external features of development of the Chimaeroid fish, *Callorhinchus milii* (Holocephali, Callorhinchidae). *J. Morphol.,* **236**, 25-47.

Diez, J.M. and Davenport, J. (1990). Energy exchange between the yolk and embryo of dogfish (*Scyliorhinus canicula* L.) eggs held under normoxic, hypoxic and transient anoxic conditions. *Comp. Biochem. Physiol.* B, **96**, 825-830.

Duncan, W.P., Silva, N.F., and Fernandes, M.N. (2011). Mitochondrion-rich cells distribution, Na^+/K^+-ATPase activity and gill morphometry of the Amazonian freshwater stingrays (Chondrichthyes: Potamotrygonidae). *Fish Physiol. Biochem.,* **37**(3), 523-531.

Emrich, S.J., Barbazuk, W.B., Li, L., and Schnable, P.S. (2007). Gene discovery and annotation using LCM-454 transcriptome sequencing. *Genome Res.,* **17**(1), 69-73.

Esbaugh, A.J. and Walsh, P.J. (2009). Identification of two glucocorticoid response elements in the promoter region of the ubiquitous isoform of glutamine synthetase in gulf toadfish, *Opsanus beta*. *Am. J. Physiol. Regul. Integr. Comp. Physiol.*, **297**(4), R1075-R1081.

Essex-Fraser, P.A., Steele, S.L., Bernier, N.J., Murray, B.W., Stevens, E.D., and Wright, P.A. (2005). Expression of four glutamine synthetase genes in the early stages of development of rainbow trout (*Oncorhynchus mykiss*) in relationship to nitrogen excretion. *J. Biol. Chem.,* **280**, 20268-20273.

Evans, D.H. (1981). The egg case of the oviparous elasmobranch, *Raja erinacea*, does osmoregulate. *J. Exp. Biol.,* **92**, 337-340.

Evans, D.H. (1993). Osmotic and ionic regulation. In: Evans, D.H. (Ed.) The Physiology of Fishes, CRC Press, Boca Raton, pp. 315–341.

Evans, D.H., Oikari, A., Kormanik, G.A., and Mansberger, L. (1982). Osmoregulation by the prenatal spiny dogfish, *Squalus acanthias*. *J. Exp. Biol.,* **101**(1), 295-305.

Fänge, R. and Fugelli, K. (1962). Osmoregulation in chimæroid fishes. *Nature,* **196**, 689.

Felinski, E.A., Cox, A.E., Phillips, B.E., and Antonetti, D.A. (2008). Glucocorticoids induce transactivation of tight junction genes occludin and claudin-5 in retinal endothelial cells via a novel *cis*-element. *Exp. Eye Res.,* **86**(6), 867-878.

Felipo, V. and Butterworth, R.F. (2002). Neurobiology of ammonia. *Prog. Neurobiol.,* **67**, 259-279.

Fines, G.A., Ballantyne J.S., and Wright, P.A. (2001). Active urea transport and an unusual basolateral membrane composition in the gills of a marine elasmobranch. *Am. J. Physiol. Regul. Integr. Comp. Physiol.,* **280**(1), R16-R24.

Foulley, M.M. and Mellinger, J. (1980). Étude chronologique, structurale et biométrique de l'œuf et de son développement chez la petite roussette (*Scyliorhinus canicula*) élevée en eau de mer artificielle. *Reprod. Nutr. Dev.,* **20**(6), 1835-1848.

Gautier, C., Husson, A., and Vailiant, R. (1977). Effets des glucocorticostéroïdes sur l'activité des enzymes du cycle de l'urée dans le foie foetal de rat. *Biochimie*, **59**(1), 91-95.

Gelsleichter, J. and Evans A.N. (2012), HORMONAL REGULATION OF ELASMOBRANCH PHYSIOLOGY. In: Carrier, J. Musick J. and Heithaus M. (Eds.), Biology and Ecology of Sharks and Their Relatives, CRC Press, Boca Raton, FL.

Gerst, J.W. and Thorson, T.B. (1977). Effects of saline acclimation on plasma electrolytes, urea excretion, and hepatic urea biosynthesis in a freshwater stingray, *Potamotrygon* sp. garman, 1877. *Comp. Biochem. Physiol.* A, **56**(1), 87-93.

Giannopoulos, G. (1975). Ontogeny of glucocorticoid receptors in rat liver. *J. Biol. Chem.,* **250**(15), 5847-5851.

Gill, H.S. and Eisenberg, D. (2001). The crystal structure of phosphinothricin in the active site of glutamine synthetase illuminates the mechanism of enzymatic inhibition.

Biochemistry, **40**, 1903-1912.

Goldstein, L. and Forster, R.P. (1971). Osmoregulation and urea metabolism in the little skate *Raja erinacea*. *Am. J. Physiol.*, **220**(3), 742-746.

Gordon, M.S., Schmidt-Nielsen, K., and Kelly, H.M. (1961). Osmotic regulation in the crab-eating frog (*Rana cancrivora*). *J. Exp. Biol.,* **38**(3), 659-678.

Gotoh, T., Chowdhury, S., Takiguchi, M., and Mori, M. (1997). The Glucocorticoid-responsive Gene Cascade: Activation of the rat arginase gene through induction of C/EBPβ. *J. Biol. Chem.*, **272**(6), 3694-3698.

Greenaway, P. and Nakamura, T. (1991). Nitrogenous excretion in two terrestrial crabs (*Gecarcoidea natalis* and *Geograpsus grayi*). *Physiol. Zool.* **64**(3), 767-786.

Griffith, R.W. (1991). Guppies, toadfish, lungfish, coelacanths and frogs: a scenario for the evolution of urea retention in fishes. *Env. Biol. Fish.,* **32**, 199-218.

Grody, W.W., Argyle, C., Kern, R.M., Dizikes, G.J., Spector, E.B., Strickland, A.D., Klein, D., and Cederbaum, S.D. (1989). Differential expression of the two human arginase genes in hyperargininemia. Enzymatic, pathologic, and molecular analysis. *J. Clin. Invest.,* **83**, 602-609.

Grogan, E.D., Lund, R., and Greenfest-Allen, E. (2004). The origin and relationships of early chondrichthyans. In: Carrier J.C., Musick J.A., and Heithanus M.R. (Eds.) Biology of sharks and their relatives, CRC Press, Boca Raton, FL, pp. 3–31

Haggerty, D.F., Spector, E.B., Lynch, M., Kern, R., Frank, L.B., and Cederbaum, S.D. (1983). Regulation of expression of genes for enzymes of the mammalian urea cycle in permanent cell-culture lines of hepatic and non-hepatic origin. In Enzyme Induction and Modulation (pp. 57-76). Springer US.

Hamlett, W.C. and Koob, T.J. (1999). Female reproductive system. In: Hamlett, W.C. (Ed.), Sharks, skates, and rays the biology of elasmobranch fishes, The Johns Hopkins University Press, London, pp. 398-433.

Hazon, N., and Henderson, I.W. (1984). Secretory dynamics of 1 apha-hydroxycorticosterone in the elasmobranch fish, *Scyliorhinus canicula*. *J. Endocrinol.,* **103**(2), 205-211.

Hazon, N., Tierney, M.L., Anderson, G., Mackenzie, S., Cutler, C., and Cramb, G. (1997). Ion and water balance in elasmobranch fish. In: Hazon, N., Eddy, F.B., Flik, G. (Eds.), Ionic Regulation in Animals, Springer, Berlin, pp. 70-86.

Hazon, N., Wells, A., Pillans, R.D., Good, J.P., Anderson, G.W., and Franklin, C.E. (2003). Urea based osmoregulation and endocrine control in elasmobranch fish with special reference to euryhalinity. *Comp Biochem. Physiol.* B, **136**(4), 685-700.

Hazra, A., DuBois, D.C., Almon, R.R., Snyder, G.H., and Jusko, W.J. (2008). Pharmacodynamic modeling of acute and chronic effects of methylprednisolone on hepatic urea cycle genes in rats. *Gene Regul Syst. Biol.,* **14**(2), 1-19.

Hinegardner, R. (1976). The cellular DNA content of sharks, rays and some other fishes. *Comp. Biochem. Physiol.* B, **55**, 367-370.

Hiroi, J., Kaneko, T., Seikai, T., and Tanaka, M. (1998). Developmental sequence of chloride cells in the body skin and gills of Japanese flounder (*Paralichthys olivaceus*) larvae. *Zool. Sci.,* **15**, 455-460.

Hiroi, J., Kaneko, T., and Tanaka, M. (1999). *In vivo* sequential changes in chloride cell morphology in the yolk-sac membrane of Mozambique tilapia (*Oreochromis mossambicus*) embryos and larvae during seawater adaptation. *J. Exp. Biol.,* **202**(24), 3485-3495.

Hiroi, J., Yasumasu, S., McCormick, S.D., Hwang, P.P., and Kaneko, T. (2008). Evidence for an apical Na-Cl cotransporter involved in ion uptake in a teleost fish. *J. Exp. Biol.,* **211**, 2584-2599.

Hoff, G.R. (2008). A nursery site of the Alaska skate (*Bathyraja parmifera*) in the eastern Bering Sea. *Fish. Bull.,* **106**(3), 233-244.

Holt, W.F. and Idler, D.R. (1975). Influence of the interrenal gland on the rectal gland of a skate. *Comp Biochem. Physiol.* C, **50**(1), 111.

Hong, J., Salo, W.L., Lusty, C.J., and Anderson, P.M. (1994). Carbamyl phosphate synthetase III, an evolutionary intermediate in the transition between glutamine-dependent and ammonia-dependent carbamyl phosphate synthetases. *J. Mol. Biol.,* **243**, 131-140.

Hornsey, D.J. (1978). Permeability coefficients of the egg-case of *Scyliorhinus canicula L.*. *Experientia,* **34**, 1596-1597.

Hu, M.Y., Tseng, Y.C., Lin, L.Y., Chen, P.Y., Charmantier-Daures, M., Hwang, P.P., and Melzner, F. (2011). New insights into ion regulation of cephalopod molluscs: a role of epidermal ionocytes in acid-base regulation during embryogenesis. *Am. J. Physiol. Regul. Integr. Comp. Physiol.,* **301**, R1700-1709.

Hughes, G.M., Perry, S.F., and Piiper, J. (1986). Morphometry of the gills of the elasmobranch *Scyliorhinus stellaris* in relation to body size. *J. Exp. Biol.*, **121**(1), 27-42.

Hunter, A. and Dauphinee, J.A. (1924). Quantitative studies concerning the distribution of arginase in fishes and other animals. *Proc. Biol. Sci.* B, **97**(682), 227-242.

Husson, A., Bouazza, M., Buquet, C., and Vaillant, R. (1983). Hormonal regulation of two urea-cycle enzymes in cultured foetal hepatocytes. *Biochem. J.*, **216**, 281-285.

Hwang, P.P. and Wu, S.M. (1993). Role of Cortisol in Hypoosmoregulation in Larvae of the Tilapia (*Oreochromis mossambicus*). *Gen. Comp. Endocrinol.*, **92**(2), 318-324.

Hyodo, S., Tsukada, T., and Takei, Y. (2004). Neurohypophysial hormones of dogfish, *Triakis scyllium*: structures and salinity-dependent secretion. *Gen. Comp. Endocrinol.,* **138**(2), 97-104.

Hyodo, S., Bell, J.D., Healy, J.M., Kaneko, T., Hasegawa, S., Takei, Y., Donald, J.A., and Toop, T. (2007). Osmoregulation in elephant fish *Callorhinchus milii* (holocephali), with special reference to the rectal gland. *J. Exp. Biol.,* **210**, 1303-1310.

Hyodo, S., Kakumura, K., Takagi, W., Hasegawa, K., and Yamaguchi, Y. (2014). Morphological and functional characteristics of the kidney of cartilaginous fishes: with special reference to urea reabsorption. *Am. J. Physiol. Regul. Integr. Comp. Physiol.*, **307**(12), R1381-R1935.

Idler, D.R. and Truscott, B. (1966). lα-Hydroxycorticosterone from cartilaginous fish: a new adrenal steroid in blood. *J. Fisheries Res. Board Canad.,* **23**, 615-619.

Inoue, J.G., Miya, M., Lam, K., Tay, B.H., Danks, J.A., Bell, J., Walker, T.I., and Venkatesh, B. (2010). Evolutionary origin and phylogeny of the modern holocephalans (chondrichthyes: chimaeriformes): A mitogenomic perspective. *Mol Biol Evol.,* **27**, 2576-2586.

Janssens, P.A. and Cohen, P.P. (1968). Biosynthesis of urea in the estivating african lungfish and in *Xenopus laevis* under conditions of water-shortage. *Comp. Biochem. Physiol.,* **24**, 887-898.

Jenkinson, C.P., Grody, W.W., and Cederbaum, S.D. (1996). Comparative properties of arginases. *Comp. Biochem. Physiol.* B, **114**, 107-132.

Joerink, M., Savelkoul, H.F., and Wiegertjes, G.F., (2006). Evolutionary conservation of alternative activation of macrophages: structural and functional characterization of arginase 1 and 2 in carp (*Cyprinus carpio L.*). *Mol. Immunol.,* **43**, 1116-1128.

Kajimura, M., Walsh, P.J., Mommsen, T.P., and Wood, C.M. (2006). The dogfish shark (*Squalus acanthias*) increases both hepatic and extrahepatic ornithine urea cycle enzyme activities for nitrogen conservation after feeding. *Physiol. Biochem. Zool.,* **79**, 602-613.

Kakumura, K., Watanabe, S., Bell, J.D., Donald, J.A., Toop, T., Kaneko, T., and Hyodo, S. (2009). Multiple urea transporter proteins in the kidney of holocephalan elephant fish (*Callorhinchus milii*). *Comp. Biochem. Physiol.* B, **154**, 239-247.

Kaneko, T., Watanabe, S., and Lee, K.M. (2008). Functional morphology of mitochondrion-rich cells in euryhaline and stenohaline teleosts. *Aqua Biosci. Monogr.*, **1**, 1-62.

Kanyo, Z.F., Scolnick, L.R., Ash, D.E., and Christianson, D.W. (1996). Structure of a unique binuclear manganese cluster in arginase. *Nature,* **383**, 554-557.

Karnaky, K.J., Jr. (1998). Osmotic and ionic regulation. In: Evans, D.H., Claibourne, J.B. (Eds.), The Physiology of Fishes, 2nd ed. CRC Press, Boca Raton, pp.157–176.

Katoh, F., Shimizu, A., Uchida, K., and Kaneko, T. (2000). Shift of chloride cell distribution during early life stages in seawater-adapted killifish, *Fundulus heteroclitus*. *Zoolog. Sci.,* **17**, 11-18.

Katoh, F. and Kaneko, T. (2003). Short-term transformation and long-term replacement of branchial chloride cells in killifish transferred from seawater to freshwater, revealed by morphofunctional observations and a newly established 'time-differential double fluorescent staining' technique. *J. Exp. Biol.*, **206**(22), 4113-4123.

Kelly, S.P. and Chasiotis, H. (2011). Glucocorticoid and mineralocorticoid receptors regulate paracellular permeability in a primary cultured gill epithelium. *J. Exp. Biol.,* **214**(14), 2308-2318.

Kikugawa, K., Katoh, K., Kuraku, S., Sakurai, H., Ishida, O., Iwabe, N., and Miyata, T. (2004). Basal jawed vertebrate phylogeny inferred from multiple nuclear DNA-coded genes. *BMC Biol.,* **2**(1), 3.

Kime, D.E. (1977). Measurement of 1α-Hydroxycorticosterone and other corticosteroids in elasmobranch plasma by radioimmunoassay. *Gen. Comp. Endocrinol.,* **33,** 344-351.

King, P.A. and Goldstein, L. (1983). Renal ammoniagenesis and acid excretion in the dogfish, *Squalus acanthias*. *Am. J. Physiol.,* **245**, R581-589.

Klinge, C.M. (2000). Estrogen receptor interaction with co-activators and co-repressors. *Steroids,* **65**, 227-251.

Kormanik, G.A. (1988). Time course of the establishment of uterine seawater conditions in late-term pregnant spiny dogfish (*Squalus acanthias*). *J. Exp. Biol.,* **137**(1), 443-456.

Kormanik, G.A. (1992). Ion and osmoregulation in prenatal elasmobranchs: evolutionary implications. *Am. Zool.,* **32**, 294-302.

Kormanik, G.A. (1993). Ionic and osmotic environment of developing elasmobranch embryos. *Env. Biol. Fish.,* **38**, 233-240.

Kormanik, G.A. and Evans, D.H. (1986). The acid-base status of prenatal pups of the dogfish, *Squalus acanthias*, in the uterine environment. *J. Exp. Biol.,* **125**, 173-179.

Kraus, J.P., Hodges, P.E., Williamson, C.L., Horwich, A.L., Kalousek, F., Williams, K.R., and Rosenberg, L.E. (1985). A cDNA clone for the precursor of rat mitochondrial ornithine transcarbamylase: comparison of rat and human leader sequences and conservation of catalytic sites. *Nucleic Acids Res.,* **13**, 943-952.

Krust, A., Green, S., Argos, P., Kumar, V., Walter, P., Bornert, J.M., and Chambon, P. (1986). The chicken oestrogen receptor sequence: homology with v-*erb*A and the human oestrogen and glucocorticoid receptors. *EMBO J.,* **5**(5), 891.

Kumai, Y., Nesan, D., Vijayan, M.M., and Perry, S.F. (2012) Cortisol regulates Na+ uptake in zebrafish, *Danio rerio*, larvae via the glucocorticoid receptor, *Mol. Cell. Endocrinol.,* **364,** 113–125.

Kumar, R. and Thompson, E.B. (1999). The structure of the nuclear hormone receptors. *Steroids*, **64**(5), 310-319.

Lambson, R.O. (1970). An electron microscopic study of the entodermal cells of the yolk sac of the chick during incubation and after hatching. *Am. J. Anat.,* **129**, 1-19.

Lamers, W.H. and Mooren, P.G. (1981). Multihormonal control of enzyme clusters in rat liver ontogenesis II. Role of glucocorticosteroid and thyroid hormone and of

glucagon and insulin. *Mech. Ageing Dev.*, **15**(1), 93-118.

Laud, P.R. and Campbell, J.W. (1994). Genetic basis for tissue isozymes of glutamine synthetase in elasmobranchs. *J. Mol. Evol.,* **39**, 93-100.

Lechenault, H., Wrisez, F., and Mellinger, J. (1993). Yolk utilization in *Scyliorhinus canicula*, an oviparous dogfish. *Env. Biol. Fish.,* **38**, 241-252.

Maisey, J.G. (1984). Higher elasmobranch phylogeny and biostratigraphy. *Zool. J. Linnean Soc.,* **82**(1-2), 33-54.

Maisey, J.G. (1986). Heads and tails: a chordate phylogeny. *Cladistics,* **2**(4), 201-256.

Matthews, G.D., Gould, R.M., and Vardimon, L. (2005). A single glutamine synthetase gene produces tissue-specific subcellular localization by alternative splicing. *FEBS Lett.,* **579**, 5527-5534.

Maxwell, E.E., Fröbisch, N.B., and Heppleston, A.C. (2008). Variability and conservation in late chondrichthyan development: ontogeny of the winter skate (*Leucoraja ocellata*). *Anat. Rec.,* (Hoboken). **291**(9), 1079-1087.

McCormick, S.D. and Bradshaw, D. (2006). Hormonal control of salt and water balance in vertebrates. *Gen. Comp. Endocrinol.*, **147**(1), 3-8.

McCormick, S.D., Regish, A., O'Dea, M.F., and Shrimpton, J.M. (2008). Are we missing a mineralocorticoid in teleost fish? Effects of cortisol, deoxycorticosterone and aldosterone on osmoregulation, gill Na^+, K^+ATPase activity and isoform mRNA levels in Atlantic salmon. *Gen. Comp. Endocrinol.,* **157**, 35-40.

McCurley, A.T. and Gallard, G.V. (2008). Characterization of housekeeping genes in zebrafish: male-female differences and effects of tissue type, developmental stage and chemical treatment. *BMC Mol. Biol.,* **9**, 102.

Meijsing, S.H., Pufall, M.A., So, A.Y., Bates, D.L., Chen, L., and Yamamoto, K.R. (2009). DNA binding site sequence directs glucocorticoid receptor structure and activity. *Science*, **324**(5925), 407-410.

Mellinger, J. (1983). Egg-case diversity among dogfish, *Scyliorhinus canicula* (L.): a study of egg laying rate and nidamental gland secretory activity. *J. Fish Biol.*, ***22***(1), 83-90.

Mellinger, J., Wrisez, F., and Alluchon-Gerard, M.-J. (1986) Developmental biology of an oviparous shark, *Scyliorhinus canicula*. In: Uyeno, T., Arai, R., Taniuchi T. and Matsuura, K. (Eds.), Indo-Pacific Fish Biology, pp. 310-332.

Miyanishi, H., Okubo, K., Nobata, S., and Takei, Y. (2012). Natriuretic peptides in developing medaka embryos: implications in cardiac development by loss-of-function studies. *Endocrinology*, **154**(1), 410-420.

Mommsen, T.P. and Walsh, P.J. (1989). Evolution of urea synthesis in vertebrates: the piscine connection. *Science,* **243**, 72-75.

Mommsen, T.P., Vijayan, M.M., and Moon, T.W. (1999). Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. *Rev. Fish Biol. Fisheries*, **9**(3), 211-268.

Morozova, O., Hirst, M., and Marra, M.A. (2009). Applications of new sequencing technologies for transcriptome analysis. *Annu. Rev. Genomics Hum. Genet.*, **10**, 135-151.

Morris, S.M. Jr. (1992). Regulation of enzymes of urea and arginine synthesis. *Annu. Rev. Nutr.*, **12**(1), 81-101.

Mulrow, P.J., Ganong, W.F., Cera, G., and Kuljian, A. (1962). The nature of the aldosterone-stimulating factor in dog kidneys. *J. Clin. Invest.,* **41**(3), 505.

Murray, B.W., Busby, E.R., Mommsen, T.P., and Wright, P.A. (2003). Evolution of glutamine synthetase in vertebrates: multiple glutamine synthetase genes expressed in rainbow trout (*Oncorhynchus mykiss*). *J. Exp. Biol.*, **206**, 1511-1521.

Mutz, K.O., Heilkenbrinker, A., Lönne, M., Walter, J.G., and Stahl, F. (2013). Transcriptome analysis using next-generation sequencing. *Curr. Opin. Biotechnol.,* **24**(1), 22-30.

Nakada, T., Westhoff, C.M., Yamaguchi, Y., Hyodo, S., Li, X., Muro, T., Kato, A., Nakamura, N., and Hirose, S. (2010). Rhesus glycoprotein p2 (Rhp2) is a novel member of the Rh family of ammonia transporters highly expressed in shark kidney. *J. Biol. Chem.,* **285**, 2653-2664.

Nakagawa, T., Lomb, D.J., Haigis, M.C., and Guarente, L. (2009). SIRT5 deacetylates carbamoyl phosphate synthetase 1 and regulates the urea cycle. *Cell,* **137**, 560-570.

Nebes, V.L. and Morris, S.M. Jr. (1988). Regulation of messenger ribonucleic acid levels for five urea cycle enzymes in cultured rat hepatocytes. Requirements for cyclic adenosine monophosphate, glucocorticoids, and ongoing protein

synthesis. *Mol. Endocrinol.*, **2**(5), 444-451.

Needham, J.N. and Needham, D.M. (1930). Nitrogen excretion in selachian ontogeny. *J. Exp. Biol.,* **7**, 7-18.

Oka, K., Kohno, S., Urushitani, H., Guillette Jr, L.J., Ohta, Y., Iguchi, T., and Katsu, Y. (2013). Molecular cloning and characterization of the corticoid receptors from the American alligator. *Mol. Cell. Endocrinol.,* **365**(2), 153-161.

Pärt, P., Wright, P.A., and Wood, C.M. (1998). Urea and Water Permeability in Dogfish (*Squalus acanthias*) Gills. *Comp. Biochem. Physiol.* A, **119**(1), 117-123.

Perkins, F.E. (1965). Incubation of fall-spawned eggs of the little skate, *Raja erinacea*. *Copeia*, 114-115.

Pickford, G.E. and Grant, F.B. (1967). Serum osmolality in the coelacanth, *Latimeria chalumnae*: urea retention and ion regulation. *Science*, **155**(3762), 568-570.

Pillans, R.D., Good, J.P., Anderson, W.G., Hazon, N., and Franklin, C.E. (2005). Freshwater to seawater acclimation of juvenile bull sharks (*Carcharhinus leucas*): plasma osmolytes and Na^+/K^+-ATPase activity in gill, rectal gland, kidney and intestine. *J. Comp. Physiol.* B, **175**(1), 37-44.

Read, L.J. (1967). Enzymes of the ornithine-urea cycle in the chimaera *Hydrolagus colliei*. *Nature,* **215**, 1412-1413.

Read, L.J. (1968a). Ornithine-urea cycle enzymes in early embryos of the dogfish *Squalus suckleyi* and the skate *Raja binoculata*. *Comp. Biochem. Physiol.,* **24**, 669-674.

Read, L.J. (1968b). Urea and trimethylamine oxide levels in elasmobranch embryos. *Biol. Bull.,* **135**, 537-547.

Ritter, N.M., Smith, D.D. Jr., and Campbell, J.W. (1987). Glutamine synthetase in liver and brain tissues of the holocephalan, *Hydrolagus colliei*. *J. Exp. Zool.,* **243**, 181-188.

Robertson, J.D. (1963). Osmoregulation and ionic composition of cells and tissues. In: Brodal, A., and Fänge, R. (Eds.), The Biology of Myxine, Scandinavian University Books, Oslo, pp. 503–515.

Robertson, J.D. (1976). Chemical composition of the body fluids and muscle of the hagfish *Myxine glutinosa* and the rabbit-fish *Chimaera monstrosa*. *J. Zool. Lond.,* **178**, 261-277.

Rodda, K.R. and Seymour, R.S. (2008). Functional morphology of embryonic development in the Port Jackson shark *Heterodontus portusjacksoni* (Meyer). *J. Fish Biol.*, **72**(4), 961-984.

Rosa, R.S., Castello, H.P., and Thorson, T.B. (1987). *Plesiotrygon iwamae*, a new genus and species of neotropical freshwater stingray (Chondrichthyes: Potamotrygonidae). *Copeia*, 447-458.

Rosewicz, S., McDonald, A.R., Maddux, B.A., Goldfine, I.D., Miesfeld, R.L., and Logsdon, C.D. (1988). Mechanism of glucocorticoid receptor down-regulation by glucocorticoids. *J. Biol. Chem.*, **263**(6), 2581-2584.

Sathiyaa, R. and Vijayan, M.M. (2003). Autoregulation of glucocorticoid receptor by cortisol in rainbow trout hepatocytes. *Am. J. Physiol. Cell Physiol.*, **284**(6), C1508-C1515.

Scammon, R.E. (1913). The development of the elasmobranch liver. I. The development of the liver ducts and gall-bladder. *Am. J. Anat.*, **14**(3), 333-350.

Scammon, R.E. (1915). The histogenesis of the selachian Liver. *Am. J. Anat.*, **17**(3), 245-315.

Schoneveld, O.J.L.M., Gaemers, I.C., Das, A.T., Hoogenkamp, M., Renes, J., Ruijter, J.M., and Lamers, W.H. (2004). Structural requirements of the glucocorticoid-response unit of the carbamoyl-phosphate synthase gene. *Biochem. J.*, **382**, 463-470.

Si-Tayeb, K., Lemaigre, F.P., and Duncan, S.A. (2010). Organogenesis and development of the liver. *Dev. Cell.*, **18**(2), 175-189.

Smith, D.D. Jr., Ritter, N.M., and Campbell, J.W. (1987). Glutamine synthetase isozymes in elasmobranch brain and liver tissues. *J. Biol. Chem.*, **262**, 198-202.

Smith, H.W. (1929). The composition of the body fluids of elasmobranchs. *J. Biol. Chem.,* **81**, 407-419

Smith, H.W. (1931). The absorption and excretion of water and salts by the elasmobranch fishes. I. Fresh water elasmobranchs. *Am. J. Physiol.*, **98**(2), 279-295.

Smith, H.W. (1936). The retention and physiological role of urea in Elasmobranchii. *Biol. Rev.,* **11**, 49–82.

Speeg, K.V. and Campbell, J.W. (1968). Formation and volatilization of ammonia gas by terrestrial snails. *Am. J. Physiol.*, **214**(6), 1392-1402.

Srivastava, S. and Ratha, B.K. (2010). Does fish represent an intermediate stage in the evolution of ureotelic cytosolic arginase I? *Biochem. Biophys. Res. Commun.,* **391**, 1-5.

Steele, S.L., Yancey, P.H. and Wright, P.A. (2004). Dogmas and controversies in the handling of nitrogenous wastes: Osmoregulation during early development in the marine little skate *Raja erinacea*; response to changes in external salinity. *J. Exp. Biol.,* **207**, 2021-2031.

Steele, S.L., Yancey, P.H. and Wright, P.A. (2005). The little skate *Raja erinacea* exhibits an extrahepatic ornithine urea cycle in the muscle and modulates nitrogen metabolism during low-salinity challenge. *Physiol. Biochem. Zool.,* **78**, 216-226.

Stolte, E.H., van Kemenade, B.L.V., Savelkoul, H.F., and Flik, G. (2006). Evolution of glucocorticoid receptors with different glucocorticoid sensitivity. *J. Endocrinol.*, **190**(1), 17-28.

Sturm, A., Bury, N., Dengreville, L., Fagart, J., Flouriot, G., Rafestin-Oblin, M.E., and Prunet, P. (2005). 11-deoxycorticosterone is a potent agonist of the rainbow trout (*Oncorhynchus mykiss*) mineralocorticoid receptor. *Endocrinology*, **146**(1), 47-55.

Takagi, W., Kajimura, M., Bell, J.D., Toop, T., Donald, J.A., and Hyodo, S. (2012). Hepatic and extrahepatic distribution of ornithine urea cycle enzymes in holocephalan elephant fish (*Callorhinchus milii*). *Comp. Biochem. Physiol.* B, **161**, 331-340.

Takagi, W., Kajimura, M., Tanaka, H., Hasegawa, K., Bell, J.D., Toop, T., Donald, J.A., and Hyodo, S. (2014). Urea-based osmoregulation in the developing embryo of oviparous cartilaginous fish (*Callorhinchus milii*): contribution of the extraembryonic yolk sac during the early developmental period. *J. Exp. Biol.,* **217**, 1353-1362.

Takabe, S., Teranishi, K., Takaki, S., Kusakabe, M., Hirose, S., Kaneko, T., and Hyodo, S. (2012). Morphological and functional characterization of a novel Na^+/K^+-ATPase-immunoreactive, follicle-like structure on the gill septum of Japanese banded houndshark, *Triakis scyllium*. *Cell Tissue Res.,* **348**, 141-153.

Takahashi, H. and Sakamoto, T. (2013). The role of 'mineralocorticoids' in teleost fish: relative importance of glucocorticoid signaling in the osmoregulation and

'central' actions of mineralocorticoid receptor. *Gen. Comp. Endocrinol.*, **181**, 223-228.

Takiguchi, M., Haraguchi, Y., and Mori, M. (1988). Human liver-type arginase gene: structure of the gene and analysis of the promoter region. *Nucleic Acids Res.*, **16**(18), 8789-8802.

Tam, W.L., Wong, W.P., Loong, A.M., Hiong, K.C., Chew, S.F., Ballantyne, J.S., and Ip, Y.K. (2003). The osmotic response of the Asian freshwater stingray (*Himantura signifer*) to increased salinity: a comparison with marine (*Taeniura lymma*) and Amazonian freshwater (*Potamotrygon motoro*) stingrays. *J. Exp. Biol.*, **206**(17), 2931-2940.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. (2013). MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.,* **30**(12), 2725-2729.

Tanaka, S., Shiobara, Y., Hioki, S., Abe, H., Nishi, G., Yano, K., and Suzuki, K. (1990). The reproductive biology of the frilled shark, *Chlamydoselachus anguineus*, from Suruga Bay, Japan. *Jan. J. Ichthyol.,* **37**(3), 273-291.

Thornton, J.W. (2001). Evolution of vertebrate steroid receptors from an ancestral estrogen receptor by ligand exploitation and serial genome expansions. *Proc. Nati. Acad. Sci.*, **98**(10), 5671-5676.

Thorson, T.B. and Gerst, J.W. (1972). Comparison of some parameters of serum and uterine fluid of pregnant, viviparous sharks (*Carcharhinus leucas*) and serum of their near-term young. *Comp. Biochem. Physiol.* A, **42**, 33-40.

Truscott, B. and Idler, D.R. (1968). The widespread occurrence of a corticosteroid lα-hydroxylase in the interrenals of Elasmobranchii. *J. Endocrinol.,* **40**, 5 15-526.

Truscott, B. and Idler, D.R. (1972). Corticosteroids in plasma of elasmobranchs. *Comp. Biochem. Physiol.* A, **42**(1), 41-50.

Utida, S., Hirano, T., Oide, H., Ando, M., Johnson, D.W., and Bern, H.A. (1972). Hormonal control of the intestine and urinary bladder in teleost osmoregulation. *Gen. Comp. Endocrinol*., **3**, 317-327.

Venkatesh, B., Tay, A., Dandona, N., Patil, J.G., and Brenner, S. (2005). A compact cartilaginous fish model genome. *Curr. Biol.,* **15**, R82-83.

Venkatesh, B., Lee, A.P., Ravi, V., Maurya, A.K., Lian, M.M., Swann, J.B., ... and

Warren, W.C. (2014). Elephant shark genome provides unique insights into gnathostome evolution. *Nature*, **505**(7482), 174-179.

Walsh, P.J., Tucker, B.C., and Hopkins, T.E. (1994). Effects of confinement/crowding on ureogenesis in the gulf toadfish *Opsanus beta*. *J. Exp. Biol.,* **191**, 195-206.

Walsh, P.J. and Mommsen, T.P. (2001). Evolutionary considerations of nitrogen metabolism and excretion. In: Wright, P.A., Anderson, P.M. (Eds.), Nitrogen Excretion, Vol. 20. Fish Physiology, Academic Press, San Diego, pp. 1-30.

Walsh, P.J., Mayer, G.D., Medina, M., Bernstein, M.L., Barimo, J.F., and Mommsen, T.P. (2003). A second glutamine synthetase gene with expression in the gills of the gulf toadfish (*Opsanus beta*). *J. Exp. Biol*., **206**, 1523-1533.

Webb, J.T. and Brown, G.W. Jr. (1980). Glutamine synthetase: assimilatory role in liver as related to urea retention in marine chondrichthyes. *Science,* **208**, 293-295.

Wells, A., Anderson, W.G., and Hazon, N. (2002). Development of an in situ perfused kidney preparation for elasmobranch fish: action of arginine vasotocin. *Am. J. Physiol. Regul. Integr. Comp. Physiol.*, **282**(6), R1636-R1642.

Weidenfeller, C., Schrot, S., Zozulya, A., and Galla, H.J. (2005). Murine brain capillary endothelial cells exhibit improved barrier properties under the influence of hydrocortisone. *Brain Res.*, **1053**(1), 162-174.

Whelan, S. and Goldman, N. (2001). A general empirical model of protein evolution derived from multiple protein families using a maximum-likelihood approach. Molecular biology and evolution, **18**(5), 691-699.

Wieser, W., Schweizer, G., and Hartenstein, R. (1969). Patterns in the release of gaseous ammonia by terrestrial isopods. *Oecologia*, **3**(3-4), 390-400.

White, W.T. and Last, P.R. (2012). A review of the taxonomy of chondrichthyan fishes: a modern perspective. *J. Fish Biol.,* **80**(5), 901-917.

Wood, C.M., Pärt, P., and Wright, P.A. (1995). Ammonia and urea metabolism in relation to gill function and acid-base balance in a marine elasmobranch, the spiny dogfish (*Squalus acanthias*). *J. Exp. Biol.*, **198**(7), 1545-1558.

Wourms, J.P. (1977). Reproduction and development in chondrichthyan fishes. *Am. Zool.*, **17**(2), 379-410.

Wourms, J.P. (1981). Viviparity: the maternal-fetal relationship in fishes. *Am.*

Zool., **21**(2), 473-515.

Wright, P.A. (1995). Nitrogen excretion: three end products, many physiological roles. *J. Exp. Biol.*, **198**(2), 273-281.

Wright, P.A., Campbell, A., Morgan, R.L., Rosenberger, A.G., and Murray, B.W. (2004). Dogmas and controversies in the handling of nitrogenous wastes: expression of arginase type I and II genes in rainbow trout: influence of fasting on liver enzyme activity and mRNA levels in juveniles. *J. Exp. Biol.,* **207**, 2033-2042.

Yadgary, L., Yair, R., and Uni, Z. (2011). The chick embryo yolk sac membrane expresses nutrient transporter and digestive enzyme genes. *Poult. Sci.*, **90**(2), 410-416.

Yamaguchi, Y., Takaki, S., and Hyodo, S. (2009). Subcellular distribution of urea transporter in the collecting tubule of shark kidney is dependent on environmental salinity. *J. Exp. Zool. A Comp. Exp. Biol.,* **311**, 705-718.

Yancey, P.H. and Somero, G.N. (1980). Methylamine osmoregulatory solutes of elasmobranch fishes counteract urea inhibition of enzymes. *J. Exp. Zool.,* **212**, 205-213.

Zettl, K.S., Sjaastad, M.D., Riskin, P.M., Parry, G., Machen, T.E., and Firestone, G.L. (1992). Glucocorticoid-induced formation of tight junctions in mouse mammary epithelial cells *in vitro*. *Proc. Natl. Acad. Sci.*, **89**(19), 9069-9073.

Zohn, I.E. and Sarkar, A.A. (2010). The visceral yolk sac endoderm provides for absorption of nutrients to the embryo during neurulation. *Birth Defects Res.. A. Clin. Mol. Teratol.,* **88**(8), 593-600.