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RESEARCH ARTICLE

Urea-based osmoregulation in the developing embryo of oviparous cartilaginous fish (*Callorhinchus milii*): contribution of the extraembryonic yolk sac during the early developmental period

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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 extrahepatic 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, the present study examined the gene expression profiles of OUC enzymes in oviparous holocephalan elephant fish (*Callorhinchus milii*) embryos. We found 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, we found that the mRNA expression of OUC enzymes was detected in the extra-embryonic YSM; the mRNA expression of cm*cpsIII* 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 cm*gs1* transcripts in the YSM supported the mRNA findings. We also found that the cm*cpsIII* transcript is localized in the vascularized inner layer of the YSM. Taken together, our 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.

KEY WORDS: Yolk sac membrane, Ornithine urea cycle enzymes, Osmoregulation, Urea synthesis, Organogenesis, 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 mmol l^{-1}) 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), composed of the following five enzymes: rate-limiting mitochondrial carbamoyl phosphate synthetase III (CPSIII), ornithine transcarbamylase (OTC), argininosuccinate synthetase, argininosuccinate lyase and arginase (ARG). Unlike mammalian OUC, mitochondrial glutamine synthetase (GS) is also an important accessory enzyme for the piscine OUC, as 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 (Anderson et al., 2005; Fänge and Fugelli, 1962). In addition, recent findings have demonstrated that several extra-hepatic organs, such as muscle, also have a functional OUC and contribute to systemic urea production (Kajimura et al., 2006; Steele et al., 2005; Takagi et al., 2012).

However, 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). In contrast, eggs of oviparous species are laid within 3 or 4 days after fertilization, with the embryos being enclosed in a tough and fibrous egg capsule in seawater 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 seawater (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 seawater 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, we used elephant fish (*Callorhinchus milii* Bory de Saint-Vincent 1823) 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

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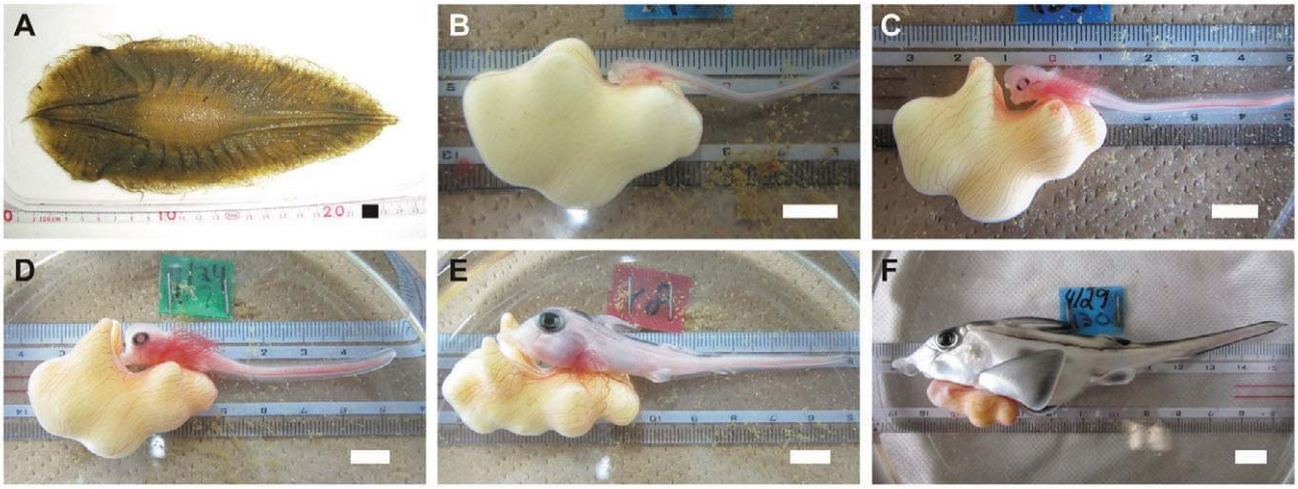

Fig. 1. An egg capsule and a series of embryos at different developmental stages of elephant fish. Developmental stages were identified according to Didier et al. (Didier et al., 1998). (A) A tough and fibrous egg capsule, which is a common feature of oviparous cartilaginous fishes. (B) Embryo at stage 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 bars, 1 cm.

fish (Venkatesh et al., 2005). We previously characterized hepatic and extra-hepatic urea production in adult elephant fish (Takagi et al., 2012). In this study, we 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.

RESULTS

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

In the present study, we broadly defined development of elephant fish embryos into three periods as described in Materials and methods: (1) the early period (stages 28–31) (Fig. 1B,C); (2) the middle period (stages 32–34) (Fig. 1D,E); and (3) the late period (from stage 35 to hatching) (Fig. 1F). 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 seawater, 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 1). 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 2). Before stage 31, we could not obtain enough body fluid for measurements because of the small size of the embryos; the urea concentration was measurable only from one fish and was found to be similar to the adult level (Table 2).

Gene expression profile of OUC enzymes in embryos during development

As 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 (Takagi et al., 2012). To this end, the total amount of the 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 we 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. 2).

For embryos of stages 33, 34 and 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. 3A–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. 3A). No significant change was observed in the hepatic CPSIII mRNA level 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. 3B,E). In contrast, the tissue distribution of GS1 and GS2 transcripts showed different patterns.

Table 1. Composition of egg capsule fluid

Stage	*N*	Osmolality (mOsm kg^{-1})	Na^+ (mmol l^{-1})	Cl^- (mequiv l^{-1})	Urea (mmol l^{-1})
28	4	1033.0±19.3	576.2±6.7	532.3±10.2	ND
29	4	1023.5±16.8	562.1±9.6	534.3±9.1	ND
30 (pre-hatching period)	5	1033.0±11.3	566.5±5.0	534.6±7.3	ND
31	6	999.3±18.7	551.4±5.7	527.2±5.1	ND
32	4	994.5±27.4	571.4±3.3	531.5±9.0	ND
Seawater		1054.0	582.3	574.0	ND

Values are means ± s.e.m. ND, not detectable.

The Journal of Experimental Biology (2014) doi:10.1242/jeb.094649

Table 2. Composition of body fluid

Stage	*N*	Osmolality (mOsm kg^{-1})	Na^+ (mmol l^{-1})	Cl^- (mequiv l^{-1})	Urea (mmol l^{-1})
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

Values are means ± s.e.m.

[1]Data from Hyodo et al., 2007. Developmental stage 36 in the present study corresponds to pre-hatching ('before hatching') fish referred by Takagi et al., 2012.

GS1 mRNA expression was observed in the liver, gut and brain, while GS2 mRNA expression was observed predominately in the muscle (Fig. 3C,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 previously reported for adult elephant fish (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. 3A), the liver size increased markedly (Fig. 3F), which, at least in part, caused the elevation in the total abundance of CPSIII and other OUC enzyme mRNAs per fish (Fig. 2).

Gene expression profile of OUC enzymes in the 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

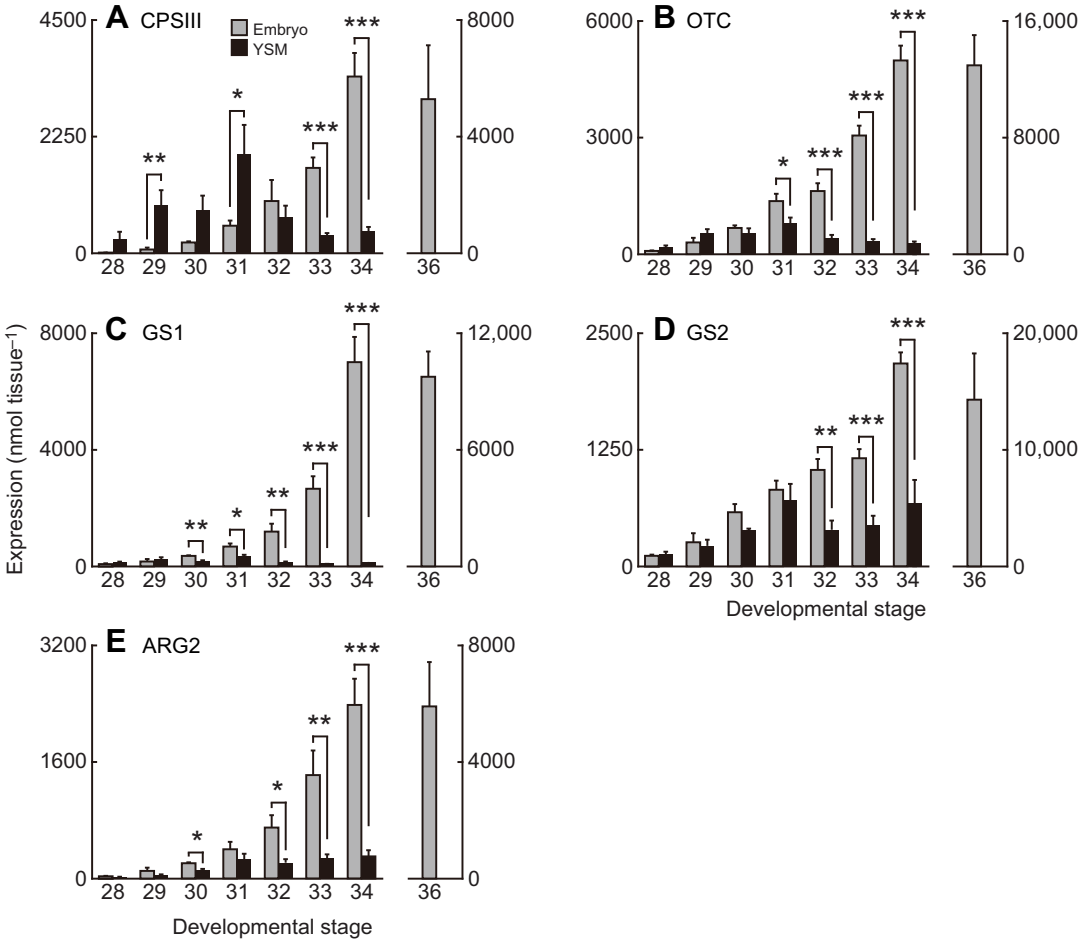

Fig. 2. Gene expression profiles of ornithine urea cycle (OUC) enzymes. Developmental changes in total mRNA abundance of CPSIII (A), OTC (B), GS1 (C), GS2 (D) and ARG2 (E) in whole embryo and the yolk sac membrane (YSM). Data are presented as means ± s.e.m. *N*=4 (developmental stage 28), 3 (stage 29), 3 (stage 30) and 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.

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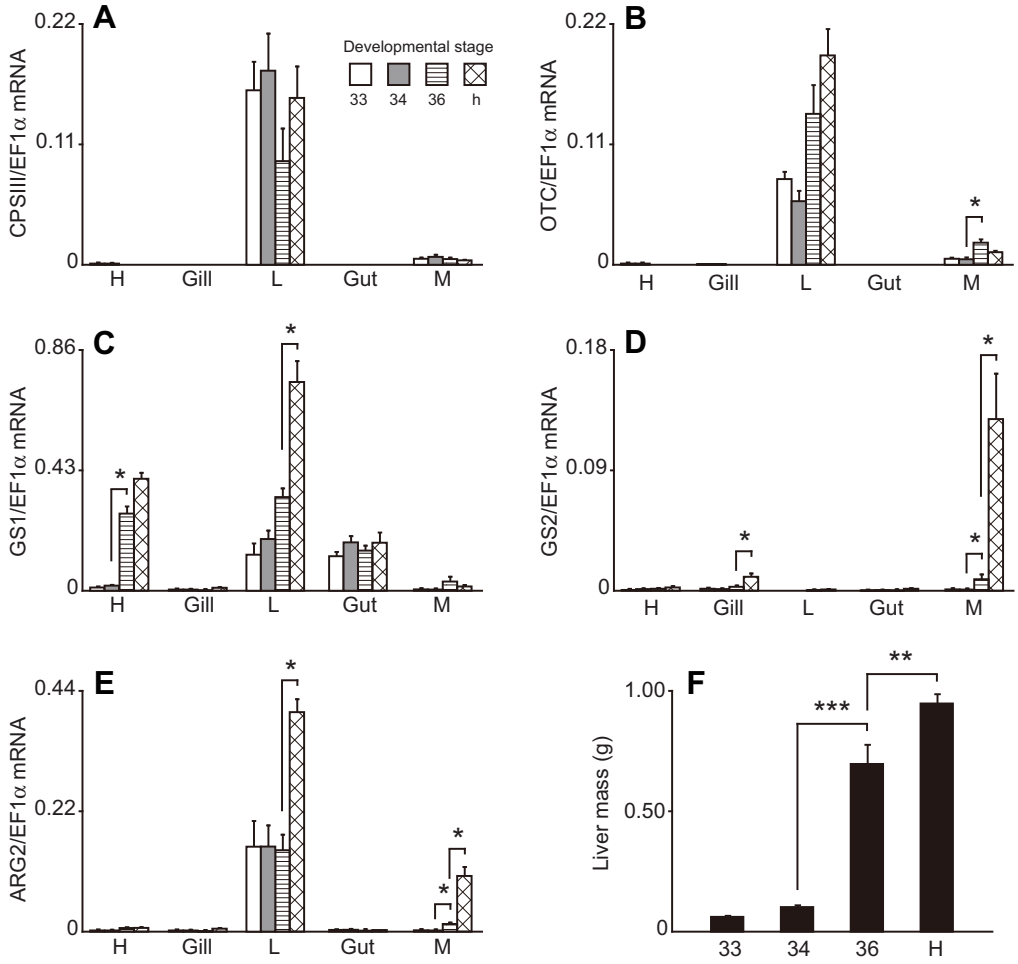

Fig. 3. Tissue distribution of OUC enzyme mRNAs in developing embryos. Developmental changes in relative mRNA levels of CPSIII (A), OTC (B), GS1 (C), GS2 (D) and ARG2 (E) in tissues of embryos. Values of each mRNA were normalized against the value of EF1α mRNA as an internal control gene. (F) Developmental increase in embryonic liver mass. All data are presented as means ± s.e.m. *N*=6 for all samples (stage 33 to hatched fish). H, head (stages 33 and 34) or brain (stage 36 and hatching); L, 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.

developmental periods are attached to a large yolk sac (Fig. 1B–E), we examined gene expression in the YSM and found expression of all OUC enzyme mRNAs (Fig. 2). 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. 2A). 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. 2B–E).

Fig. 4 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. 2A), the CPSIII mRNA level peaked at stage 30, and then subsequently decreased (Fig. 4A). 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 (Fig. 4B,C). Similarly, the expression levels of ARG2 were 10 times lower than the level in the liver (Fig. 4E).

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 is found in the brain and other organs (Takagi et al., 2012). Therefore, we examined the tissue distribution of two cm*gs1* transcripts of different sizes (GS1Long and GS1Short), together with a cm*gs2* transcript in the head, body, tail and liver (stage 34 only) of the embryonic body and in the YSM at four developmental stages (stages 28, 30, 32 and 34). As in adult fish, two transcripts of cm*gs1* of different sizes were found in developing embryos (Fig. 5A). 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. 5). 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. 5). 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. 5B). As the body samples contained the liver, it is most probable that the long transcript in these samples was derived from the liver. At stage 34, we could analyze the liver separately, and found a high expression of GS1Long in the embryonic liver (Fig. 5B), while the short-form of cm*gs1* transcript was mainly expressed in the head, tail and body, in which the liver was not included (Fig. 5A,C).

The Journal of Experimental Biology (2014) doi:10.1242/jeb.094649

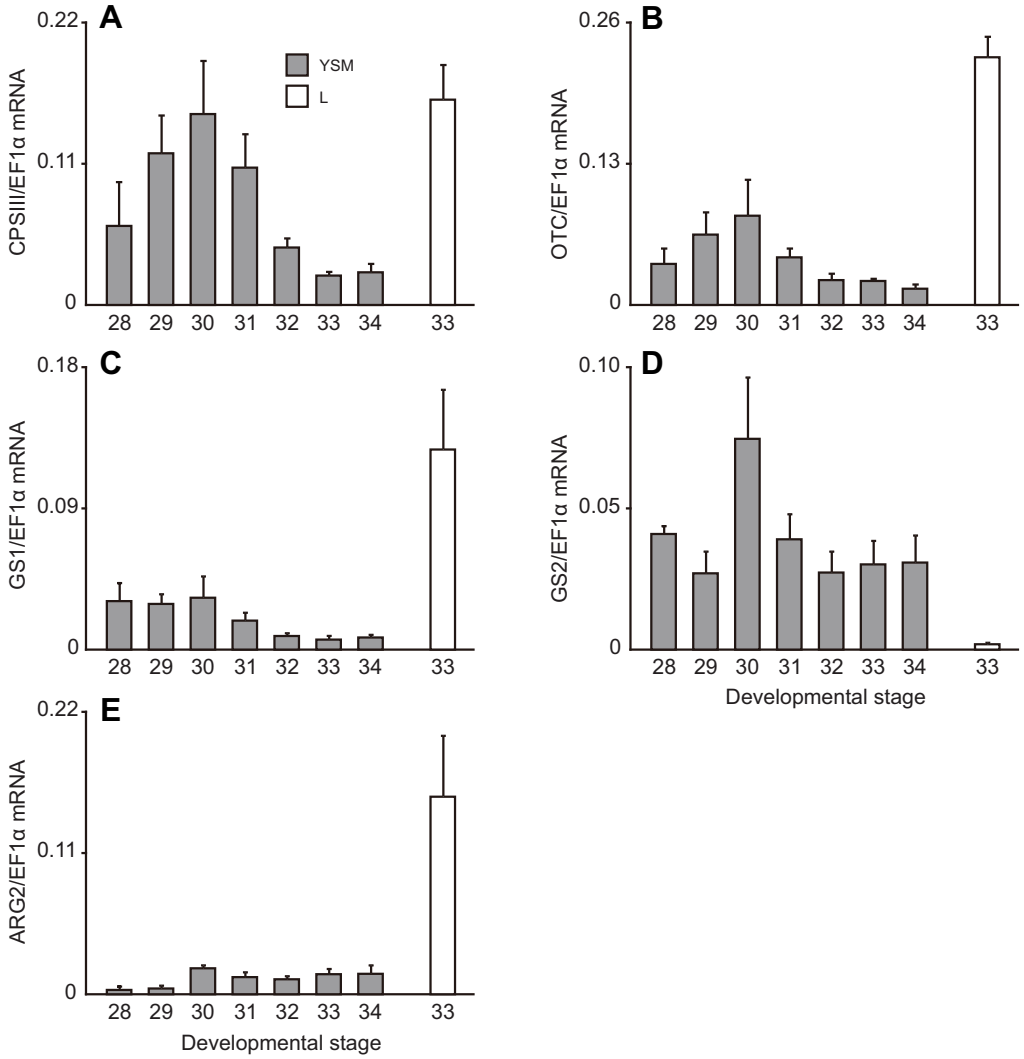

Fig. 4. OUC gene expression in YSM. Developmental changes in the relative mRNA levels of CPSIII (A), OTC (B), GS1 (C), GS2 (D) and ARG2 (E) in YSM. Each value was normalized by EF1α as described in Fig. 3. All data are presented as means ± s.e.m. *N*=4 (developmental stage 28), 3 (stage 29), 3 (stage 30) and 6 (from stage 31 to 34). For comparison, mRNA levels in the liver of stage 33 embryos are also shown (*N*=6). L, liver.

The expression of the two alternatively spliced transcripts of cmg*s1* 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 (Fig. 2A, Fig. 4A), the intensity of the band corresponding to the GS1Long mRNA was stronger than that of the GS1Short (Fig. 5A). The results of qPCR analysis revealed that the expression of GS1Long in the extra-embryonic YSM was significantly higher than in embryonic tissues (Fig. 5B) and that GS1Long/total GS1 ratio in the YSM was around 50% at stage 28 and 30. However, in the later stages (32 and 34), the expression level of the long-form transcript was decreased, resulting in a decrease in the GS1Long/total GS1 ratio (Fig. 5A,C).

Activity of OUC enzymes in the 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 3). 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 the 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. 4). When the values in the YSM were compared with those in the liver of stage 36 and adult fish (Takagi et al., 2012), CPSIII activity was 11–28% that of the liver samples. In contrast, the activities of other enzymes (GS, OTC and ARG) in the YSM were 1.7–7% of those in the liver. As a consequence, the ratio between CPSIII and OTC (CPSIII/OTC) was ~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 the YSM

Under the light microscope, the YSM was composed of two cellular layers: an ectodermal outer layer with fibrous connective tissue (FCL) and a vascularized endodermal inner layer (Fig. 6A). Consistent with the previous studies, the endodermal inner layer was closely attached to yolk cytoplasm (Lechenault et al., 1993). Lechenault et al. showed regional differences in the histological structure of the vascularized YSM wall, suggesting that the YSM is functionally differentiated depending on the area (Lechenault et al., 1993). In the present study, we sampled the proximal area of the YSM, which is situated around the yolk stalk and is richly vascularized compared with the distal area. *In situ* hybridization was performed using the YSM of stage 31 when the CPSIII transcript showed the highest expression level (Fig. 2A). 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. 6B); hybridization with the sense probe of CPSIII did not show any positive signals (Fig. 6C). Furthermore, to confirm that the expression of CPSIII mRNA in the

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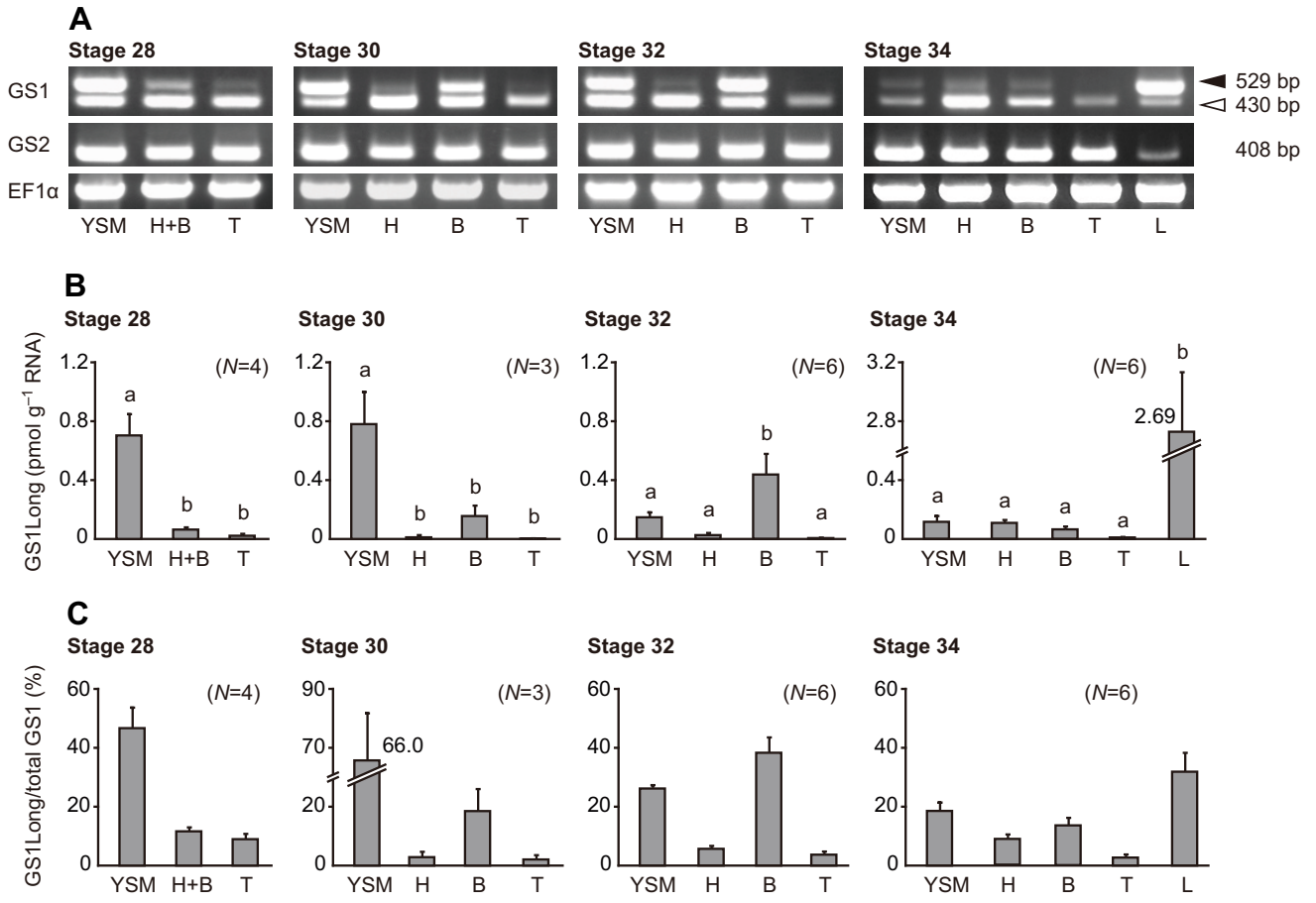

Fig. 5. Expression patterns of GS transcripts. (A) Expression patterns and developmental changes of cm*gs* transcripts (GS1Long, GS1Short and GS2) analyzed by RT-PCR. EF1α mRNA was used as a positive control for each cDNA sample. Black and white arrowheads indicate GS1Long and GS1Short mRNAs, respectively. Amplicon lengths of GS1Long, GS1Short and GS2 are also shown. (B) Expression patterns of GS1Long transcript by quantitative PCR. Within each panel, values sharing the same letter are not significantly different (P>0.05). (C) The expression ratio (percentage) of GS1Long to total GS1 (GS1Long plus GS1Short) in each tissue and its developmental changes. Note that the 'body' sample of embryo at stage 34 does not contain the liver. H, head; B, body; T, tail; L, liver.

YSM is a common phenomenon in oviparous cartilaginous fish, catshark (*Scyliorhinus torazame*) CPSIII (stCPSIII) cDNA was cloned and the localization of stCPSIII mRNA in the YSM was examined. We 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 abundantly observed only in the vascularized inner layer of the YSM (Fig. 6D).

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 the present study, we confirmed that oviparous embryos, at least after stage 31, conduct urea-based osmoregulation, and for the first time we 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. Over 80 years ago, it was reported that the egg capsule walls of the oviparous shark *S. canicula* are 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

Table 3. Activity 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

OUC, ornithine urea cycle; YSM, yolk sac membrane.
Values for enzyme activities (μmol min^{-1} g^{-1}) are presented as means ± s.e.m. N=5 (YSM at stage 31), 5 (YSM at 34) and 6 (embryonic liver at 36).
*Significant difference in CPSIII activities of YSM between stages 31 and 34 at P<0.05.
[1]Data of adult fish liver are from Takagi et al., 2012.

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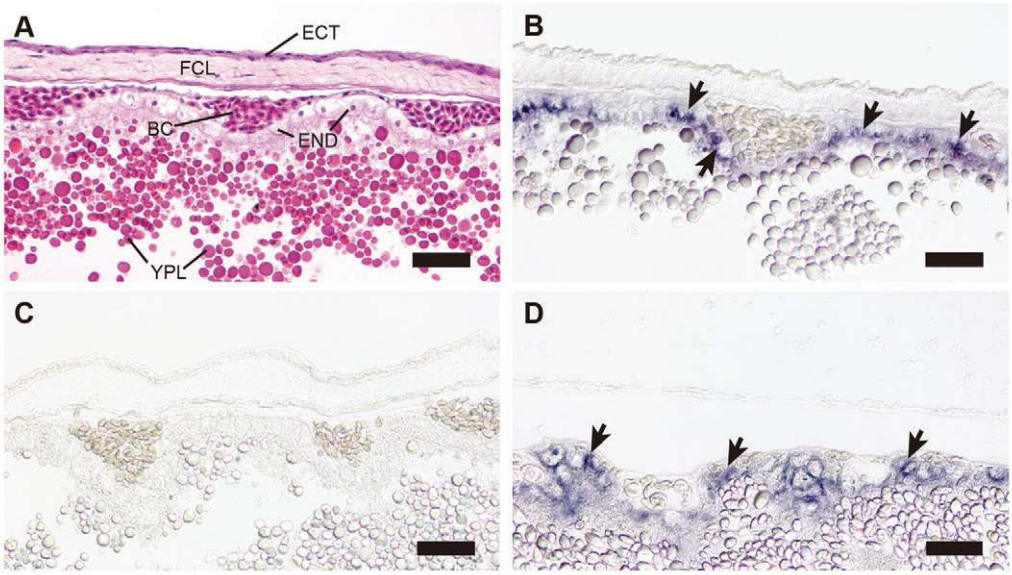

Fig. 6. Cellular localization of CPSIII mRNA. Morphological observations of the YSM shown by hematoxylin and eosin staining (A) and localization of CPSIII mRNA by *in situ* hybridization (B,C). (D) Localization of catshark (*Scyliorhinus torazame*) CPSIII mRNA confirmed the expression of CPSIII mRNA in cells of the vascularized endodermal layer of the YSM. Black arrows indicate positive signals of CPSIII mRNA in elephant fish (B) and catshark (D), respectively. ECT, ectoderm; FCL, fibrous connective layer; END, endoderm; BC, blood cell; YPL, yolk platelets. Scale bars, 50 µm.

were directly measured in *S. canicula* (Hornsey, 1978) and big skate *Raja binoculata* (Read, 1968b). More recently, it was demonstrated that the osmolality and ionic composition inside the capsule are similar to seawater within hours of oviposition (Kormanik, 1992). Conversely, Evans (Evans, 1981) reported osmotic and ionic gradients between the egg capsule fluid and surrounding seawater in oviparous little skate (*R. erinacea*), suggesting that the egg capsule of this species can be an osmotic and ionic barrier to environmental seawater. In the present study, we 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 (seawater), 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 (Read, 1968b) further elucidated that the urea concentrations in embryos and yolks are nearly equal in oviparous skate *R. binoculata*, 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, we 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 its body from the early developmental period. To our knowledge, only a few earlier studies have provided evidence that the developing embryo of oviparous cartilaginous fish produces urea. Read (Read, 1968a) demonstrated that embryos of *R. binoculata* have OTC and ARG activities, and that those enzyme activities were increased as development proceeded. More recently, research using *R. erinacea* showed the presence of CPSIII, OTC, ARG and GS activities in 4 and 8 month old embryos (Steele et al., 2004). In elephant fish, we detected the embryonic expression of mRNAs encoding a series of OUC enzymes (GS1, GS2, 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 (Takagi et al., 2012). Our 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 with 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, we focused on the mRNA expression of OUC enzymes in the YSM. Surprisingly, we found a high abundance of mRNAs encoding OUC enzymes in the 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 than in the embryonic body. As 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. We 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 cm*gs1* (GS1Long) with a 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 of 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. In contrast, the cytosolic short form of *gs1* appears to contribute to the recycling of neurotransmitters in neural tissue, and to ammonia detoxification in other extra-hepatic tissues (Matthews et al., 2005; Takagi et al., 2012). Furthermore, hybridization signals of CPSIII mRNA were found in cells comprising the vascularized endodermal layer of the YSM. This localization was further confirmed in the oviparous elasmobranch *S.*

The Journal of Experimental Biology (2014) doi:10.1242/jeb.094649

torazame, implying that the expression of CPSIII mRNA in the YSM is a common phenomenon among marine cartilaginous fish. Taken together, our findings indicate that urea is produced in the endodermal layer of the 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 the 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 seawater or freshwater environments (Ayson et al., 1994; Hiroi et al., 2008), suggesting that these extrabranchial 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 appears 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 with the liver of adult elephant fish. In general, the mRNA expression and activity of CPSIII are lower than those of 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 ~0.034 (Takagi et al., 2012), 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 ~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, we 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 (Read, 1968b) showed in *R. binoculata* that the fertilized egg and entire embryonic system (embryo plus yolk) contain notable amounts of urea during the early developmental period.

In summary, we 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.

MATERIALS AND METHODS

Embryos

In March 2011, adult elephant fish, *C. milii*, were collected in Western Port Bay, Victoria, Australia, using recreational fishing equipment consisting of a breaking strain line and a hook. Female fish were transported to Primary Industries Research Victoria, Queenscliff, in a 1000 l fish transporter. Fish were kept in a 10,000 l round tank with running seawater under a natural photoperiod for approximately 2 months. During that period, newly laid eggs were gathered from each individual (121 eggs in total), and maintained in a 1000 l tank with running seawater. 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 mass 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). We confirmed that 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.

Tissue, embryonic body fluid and egg capsule fluid sampling

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 2250 ***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°C 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, we could not dissect each organ because of 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 YSM were dissected out. After stage 36, the brain was dissected. All tissues were quickly frozen in liquid nitrogen, and then stored at −80°C. Any

remaining tissues following dissection of the above were also frozen for assessing the total abundance of target gene transcripts in the whole body.

cDNA synthesis and RT-PCR

Total RNA (2 µg) was extracted from frozen tissues by the 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 cm*gs1* and cm*gs2* (Takagi et al., 2012). The primer set for cm*gs1* is able to amplify both the long-form and short-form GS mRNAs. Thirty-two cycles of amplification were carried out. 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

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 cm*cpsIII* (GenBank accession no. AB603761), cm*otc* (AB622984), cm*gs1* (AB622985), cm*gs2* (AB622986) and cm*arg2* (AB622987), which we designed previously (Takagi et al., 2012). In the present study, we designed a new primer set that was specific for the long-form transcript of cm*gs*1. A 2 µl sample of cDNA template was 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 of known concentration were serially diluted and used as the standard templates. Total copy numbers of mRNA (nmol tissue^{-1}) in whole embryos and YSM were then calculated for absolute quantification. The amount of short-form transcript of cm*gs1* was calculated by subtracting the long-form transcript from the total cm*gs1* transcript. Elephant fish elongation factor 1α (cm*ef1α*, 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 cm*cpsIII*, cm*otc*, cm*gs1*, cm*gs1* long-form, cm*gs2*, cm*arg2* and cm*ef1α*, respectively.

In situ hybridization

Whole yolk sac was fixed in modified Bouin's fixative without glacial acetic acid, at 4°C for 24 h. 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 cm*cpsIII* fragment (1110 bp) was amplified with a gene-specific primer set, using GGTTACCCCTGTTTACTGAGG as the sense primer and CCGATAATGATACAGACTGGT as the 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 and embryos as previously described (Kajimura et al., 2006; Mommsen and Walsh, 1989; Barber and Walsh, 1993). 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 adjusted with NaOH to pH 7.5 at 22°C), and sequentially centrifuged at 8000 ***g*** for 20 min at 4°C. The supernatant fraction was used to measure the activity of GS (assayed via the formation of γ-glutamyl hydroxamate; EC 6.3.1.2), CPSIII (EC 6.3.5.5), OTC (EC 2.1.3.3) and ARG (EC 3.5.3.1).

Statistical analysis

Data are represented as means ± s.e.m. 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 mass 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 embryonic tissues. *P*-values less than 0.05 were considered statistically significant.

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Competing interests

The authors declare no competing financial interests.

Author contributions

W.T., T.T., J.A.D. and S.H. helped design the study. W.T. performed most of the experiments, and wrote the first draft. M.K. measured the enzyme activity. K.H. measured the ion levels by ion chromatography and by atomic-absorption spectrophotometry. J.D.B. and H.T. cultivated and provided elephant fish and catshark embryos, respectively. All authors contributed to the analyses and interpretation of the data.

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References

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

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.

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.

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**, 318-336.

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**, 1088-1098.

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

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

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.* **96B**, 825-830.

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

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

Hamlett, W. C. and Koob, T. J. (1999). Female reproductive system. In: *Sharks, Skates and Rays the Biology of Elasmobranch Fishes* (ed. W. C. Hamlett), pp. 398-433. London: The Johns Hopkins University Press.

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. *Zoolog. Sci.* **15**, 455-460.
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.
Hornsey, D. J. (1978). Permeability coefficients of the egg-case membrane 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.* **301**, R1700-R1709.
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.
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.
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.
Kaneko, T., Watanabe, S. and Lee, K. M. (2008). Functional morphology of mitochondrion-rich cells in euryhaline and stenohaline teleosts. *Aqua-BioScience Monographs* **1**, 1-62.
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.
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. *Environ. Biol. Fishes* **38**, 233-240.
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.
Lechenault, H., Wrisez, F. and Mellinger, J. (1993). Yolk utilization in Scyliorhinus canicula, an oviparous dogfish. *Environ. Biol. Fishes* **38**, 241-252.
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.
Mommsen, T. P. and Walsh, P. J. (1989). Evolution of urea synthesis in vertebrates: the piscine connection. *Science* **243**, 72-75.
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.
Needham, J. N. and Needham, D. M. (1930). Nitrogen excretion in selachian ontogeny. *J. Exp. Biol.* **7**, 7-18.
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.
Smith, H. W. (1936). The retention and physiological role of urea in Elasmobranchii. *Biol. Rev. Camb. Philos. Soc.* **11**, 49-82.
Steele, S. L., Yancey, P. H. and Wright, P. A. (2004). Dogmas and controversies in the handling of nitrogenous wastes: osmoregulation during early embryonic 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.
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.
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.* **161B**, 331-340.
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.* **42A**, 33-40.
Venkatesh, B., Tay, A., Dandona, N., Patil, J. G. and Brenner, S. (2005). A compact cartilaginous fish model genome. *Curr. Biol.* **15**, R82-R83.
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.
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.
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**, 593-600.