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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Keywords: Yolk sac membrane; Ornithine urea cycle enzymes; Osmoregulation, Urea synthesis; Organogenesis; Oviparous cartilaginous fish

SUMMARY

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 extraembryonic 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 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.

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, argininosuccinate lyase, 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 (Anderson et al., 2005; Fänge and Fugelli, 1962). On the other hand, recent findings have demonstrated that several extrahepatic 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).

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 pregnant, and thus requirements for ionoregulation and osmoregulation by the embryos are considered to be minimal during early development (Thorson and Gerst, 1972; Kormanik, 1993). On the other hand, 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, we 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). We previously characterized hepatic and extrahepatic 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 extraembryonic 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). We

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, we 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 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. 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

159 by a real-time quantitative PCR (qPCR) method using a 7900HT Sequence Detection
160 System (Life Technologies, Carlsbad, CA, USA). PCR reactions were performed
161 with KAPA SYBR Fast qPCR kit (Kapa Biosystems) and primer sets of cm*cpsIII*
162 (GenBank accession no. AB603761), cm*otc* (AB622984), cm*gs1* (AB622985), cm*gs2*
163 (AB622986), cm*arg2* (AB622987), which we designed previously (Takagi et al.,
164 2012). In the present study, we designed a new primer set that was specific for the
165 long-form transcript of cm*gs*1. Two μL of complementary DNA templates were
166 added to 8 μL of reaction mixture, and measurement was performed in duplicate. To
167 generate a standard curve, plasmids containing partial cDNA fragments of target genes
168 with known concentration were serially diluted and used as the standard templates.
169 Total copy numbers of mRNA (nmol/tissue) in whole embryos and YSM were then
170 calculated for absolute quantification. The amount of short-form transcript of cm*gs1*
171 was calculated by subtracting the long-form transcript from the total cm*gs1* transcript.
172 Elephant fish elongation factor 1α (cm*ef1α*, AB622989) was used as an endogenous
173 expression control to calculate relative expression values. The efficiencies for each
174 reaction were 96.5%, 96.0%, 96.4%, 93.6%, 99.5%, 93.2%, 98.6% for cm*cpsIII*,
175 cm*otc*, cm*gs1*, cm*gs1* long-form, cm*gs2*, cm*arg2*, cm*ef1α*, respectively.
176
177 ***In situ* hybridization**
178 Whole yolk sac was fixed in modified Bouin's fixative without glacial acetic acid,
179 at 4 ºC for 24h. The YSM was dissected from fixed yolk sac, washed three times with
180 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 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 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 (20mmol L^{-1} K_2HPO_4, 10mmol L^{-1} HEPES, 0.5 mmol L^{-1} EDTA, 1mmol L^{-1} dithiothreitol, 50% glycerol adjusted with NaOH to pH 7.5 at 22°C), and sequentially centrifuged at 8,000 *g* for 20 min at 4°C. The supernatant fraction was used to measure the activity of GS (assayed via the formation of g-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). The 'EC' followed by numbers is the Enzyme Commission number (EC number), which is the enzyme classification scheme recommended by the International Union of Biochemistry and Molecular Biology (IUBMB).

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, we 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. 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 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 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 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 2).

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

238 determine putative urea production sites, using previously established protocols
239 (Takagi et al., 2012). To this end, the total amount of target gene transcripts was
240 determined for whole embryos (without yolk sac), and calculated as nmol transcript
241 per fish. At stage 28, which was the first stage we sampled, the expression of all
242 transcripts encoding the OUC enzymes (CPSIII, OTC, GSs and ARG2) was already
243 detectable, and the expression consistently increased throughout development toward
244 the hatching period (Fig. 2).

245 For embryos of stages 33, 34, 36 and hatched fish, the mRNA levels of the OUC
246 enzymes were examined in head (brain for stage 36 and hatched fish), gill, liver and
247 muscle (Fig. 3A-E). In this experiment, the mRNA level of the OUC enzymes was
248 normalized against the mRNA levels of elongation factor 1 alpha (EF1α). A high
249 mRNA expression level of the rate-limiting CPSIII was observed only in the liver,
250 while a low level of mRNA expression was detected in the muscle (Fig. 3A). No
251 significant change was observed in the hepatic CPSIII mRNA levels during
252 development. Similar results were observed for both OTC and ARG2 mRNAs,
253 except that the mRNA expression levels were increased in stage 36 and/or hatched
254 fish; the changes were statistically significant for OTC mRNA in muscle and ARG2
255 mRNA in the liver and muscle (Fig. 3B, E). On the other hand, the tissue distribution
256 of GS1 and GS2 transcripts showed different patterns. GS1 mRNA expression was
257 observed in the liver, gut and brain, while GS2 mRNA expression was observed
258 predominately in the muscle (Fig. 3C, D). The expression of GS1 and GS2 mRNAs
259 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 enzymes mRNAs per fish (Fig. 2).

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. 1B-E), we examined gene expression in the yolk sac membrane (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).

Figure 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 (Figs 4B and 4C). 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 was found in the brain and other organs (Takagi et al., 2012). Therefore, we examined the tissue distribution of two cm*gs1* transcripts with 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

304 developmental stages (stage 28, 30, 32 and 34). As in adult fish, two transcripts of
305 cm*gs1* with different sizes were found in developing embryos (Fig. 5A). At stage 28,
306 where the embryonic body was separated into head/body and tail parts, a low but
307 observable amount of long transcript was found in the head/body, while expression of
308 the short transcript was predominant in the tail (Fig. 5). For stages 30 and 32,
309 embryos were separated into three parts: head, body and tail. The intense band of
310 long *gs1* transcript was detected in the 'body' samples, while the short *gs1* mRNA was
311 predominantly expressed in the head and tail samples (Fig. 5). These expression
312 patterns of the long-form and the short-form GS1 transcripts were confirmed by
313 quantitative PCR; at stage 32, the expression of GS1Long mRNA in the 'body' was the
314 highest amongst the embryonic tissues (Fig. 5B). Since the 'body' samples contained
315 the liver, it is most probable that the long transcript in the 'body' samples was derived
316 from the liver. At stage 34, we could analyze the liver separately, and found a high
317 expression of GS1Long in the embryonic liver (Fig. 5B), while the short-form of
318 cm*gs1* transcript was mainly expressed in the head, tail, and 'body', in which the liver
319 was not included (Fig. 5A, C).

320 The expression of the two alternatively spliced transcripts of cm*gs1* was also
321 observed in the YSM. During the early developmental period (stages 28 and 30), in
322 which the expression of CPSIII was high in the YSM (Figs 2A and 4A), the intensity
323 of the band corresponding to the GS1Long mRNA was stronger than that of the
324 GS1Short (Fig. 5A). The results of qPCR analysis revealed that the expression of
325 GS1Long in the extraembryonic YSM was significantly higher than embryonic tissues

(Fig. 5B) and that GS1Long/total GS1 ratio in YSM was around 50% at stage 28 and 30. On the other hand, 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. 5A, 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 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 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), the values of CPSIII activity were 11 to 28% of the liver samples. On the other hand, 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. 6A). 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, we 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. 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 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 only abundantly observed in the vascularized inner layer of YSM (Fig. 6D).

DISCUSSION

In adult cartilaginous fish, past research has revealed the contribution of hepatic and extrahepatic 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. Almost 80 years ago, it was reported that the egg capsule walls of the oviparous shark *S. 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 *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 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 (*R. erinacea*), suggesting that the egg capsule of this species can be an osmotic and ionic barrier to environmental SW. 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 (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 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 their 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 produce urea. 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 (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

414 the primary organ for urea production in embryos from at least stage 33 as well as in
415 adult fish (Takagi et al., 2012). Our results imply that the development of the liver
416 contributes to the increase in OUC mRNA abundance in the whole embryo. However,
417 during the early developmental period (stages 28-31), the abundance of OUC enzyme
418 mRNAs in the embryonic body was low compared to that of the later stages.

419 During the early developmental period, yolk is the largest mass in the egg capsule,
420 and the YSM surrounds the yolk. Therefore, we focused on mRNA expression of
421 OUC enzymes in the YSM. Surprisingly, we found a high abundance of mRNAs
422 encoding OUC enzymes in YSM during the early developmental period (stages 28-31).
423 In particular, the mRNA levels of the rate-limiting CPSIII were much higher in the
424 YSM compared to the embryonic body. Since the activities of the OUC enzymes can
425 be modulated by post-translational regulation (Nakagawa et al., 2009), the enzyme
426 activities of CPSIII, OTC, ARG and GS were also examined. We confirmed that all
427 of these enzymes are functional in the YSM throughout development; the
428 developmental changes in CPSIII activity corresponded with the changes in CPSIII
429 mRNA expression. These results indicate a considerable contribution of the YSM to
430 urea homeostasis of embryos during early development. A high expression of the
431 long form variant of cm*gs1* (GS1Long) with a mitochondrial-targeting signal (MTS)
432 also supports the existence of a functional OUC in the YSM. The long form
433 transcript of *gs1*, in which an additional exon encoding the MTS is inserted, is
434 expressed in the adult liver, and contributes to piscine OUC by donating glutamine as a
435 substrate of mitochondrial CPSIII in cartilaginous fish (Webb and Brown, 1980;

436 Takagi et al., 2012). The long form transcript of *gs1* was also predominantly
437 expressed in the embryonic liver of elephant fish early in development, and most
438 likely contributes to hepatic urea production in embryos. On the other hand, the
439 cytosolic short form of *gs1* appears to contribute to the recycling of neurotransmitters
440 in neural tissue, and for ammonia detoxification in other extrahepatic tissues
441 (Matthews et al., 2005; Takagi et al., 2012). Furthermore, hybridization signals of
442 CPSIII mRNA were found in cells comprising the vascularized endodermal layer of
443 YSM. This localization was further confirmed in the oviparous elasmobranch, *S.*
444 *torazame*, implying that the expression of CPSIII mRNA in the YSM is a common
445 phenomenon among marine cartilaginous fish. Taken together, our findings indicate
446 that urea is produced in the endodermal layer of YSM, and is then excreted into nearby
447 blood vessels, and subsequently transferred to the embryo in order to maintain a high
448 concentration of urea in the body fluid.

449 Yolk sacs are found in many vertebrates, and the extraembryonic YSM has been
450 considered to play a key role in absorption of yolk nutrients (Lambson, 1970; Diez and
451 Davenport, 1990; Lechenault et al., 1993; Zohn and Sarkar, 2010; Bauer et al., 2012).
452 An intriguing finding on the role of the YSM in teleost fish is that it is involved in
453 osmoregulation during early development (Kaneko et al., 2008). Mitochondria-rich
454 ionocytes, which are responsible for active ionic regulation in adult gill epithelia, can
455 be observed in the YSM during the early development of embryos reared in either SW
456 or FW environments (Ayson et al., 1994; Hiroi et al., 2008), suggesting that these
457 extrabranchial ionocytes are important for body fluid homeostasis of the developing

458 embryos of teleosts. The presence of ionocytes involved in ion and acid-base
459 regulation in the YSM has also been reported in squid *Sepioteuthis lessoniana* and
460 cuttlefish *Sepia officinalis* (Hu et al., 2011). Although the origin of ionocytes and
461 endodermal urea-producing cells appear to be different, the present finding that
462 cartilaginous fish YSM expresses a functional OUC strongly suggests that the
463 extraembryonic YSM of aquatic animals makes an important contribution to
464 environmental adaptation in general.

465 One of the interesting observations in the YSM is that the relative mRNA
466 expression and activity of CPSIII to other enzymes were considerably higher in the
467 YSM compared to the liver of adult elephant fish. In general, the mRNA expression
468 and activity of CPSIII is lower than the other OUC enzymes, such as OTC and ARG,
469 and thus CPSIII has been referred to as a rate-limiting enzyme in the piscine OUC
470 (Janssens and Cohen, 1968). In the liver of adult elephant fish, the ratio between
471 CPSIII and OTC activities (CPSIII/OTC) was approximately 0.034 (Takagi et al.,
472 2012), and this value is consistent with the values calculated for other species
473 (Kajimura et al., 2006). In contrast, in the YSM of elephant fish, the CPSIII/OTC
474 activity ratio was approximately 0.37 (stage 31) and 0.17 (stage 34). The high ratio
475 obtained in the YSM most probably reflects the high relative activity of CPSIII in the
476 YSM *in vivo*. Further research is necessary to clarify why such a high activity of
477 CPSIII to other OUC enzymes is required in the YSM, and whether the high ratio is a
478 common phenomenon in oviparous cartilaginous fishes.

479 In the present study, we examined embryos from stage 28 because at the earlier

480 stages of development the embryos were too small for analysis. Further research is
481 necessary to investigate how the YSM forms and at what stage the endodermal cells
482 begin to express OUC enzyme transcripts. The contribution of maternal OUC
483 enzyme mRNAs and/or maternal urea in yolk to embryonic osmoregulation during the
484 initial developmental period is also of interest. Read (1968b) showed in *R.*
485 *binoculata* that the fertilized egg and entire embryonic system (embryo plus yolk)
486 contain notable amounts of urea during the early developmental period.

487 In summary, we found that the embryos of holocephalan elephant fish conduct
488 urea-based osmoregulation from the early stages of development, and that the YSM
489 most probably makes an important contribution to urea production, particularly during
490 the early developmental period in which the liver of the embryo is not sufficiently
491 developed. After stage 32, the abundance of OUC enzyme mRNAs was dramatically
492 increased in the liver as hatching approached, while the abundance of OUC enzyme
493 mRNAs in the YSM decreased, which reflects both the decrease in expression level in
494 the YSM and the regression of the yolk sac. These results imply a functional shift of
495 the urea production site during embryonic development from the YSM to the
496 embryonic body (liver) at around stage 32. In teleosts, functional ionocytes are
497 distributed in the YSM during early embryonic stages (Kaneko et al., 2008), and a
498 distributional shift of ionocytes from the YSM to the gills occurs (Hiroi et al., 1998;
499 Katoh et al., 2000). Similar ontogeny-dependent shifts in the site of osmoregulation
500 have also been reported in marine crustaceans (Cieluch et al., 2005) and cephalopods
501 (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.

ACKNOWLEDGEMENTS

We thank Ms. Elizabeth McGrath and Mr. Rod Watson of VMSC, and Ms. Camila Martin of Monash University, Australia, for their kind support to collect samples.

FUNDING

This study was supported by a Grant-in-Aid for Scientific Research (C) and a Japan-Australia Research Cooperative Program from the Japan Society for the Promotion of Science to SH, and a Grant-in-Aid for JSPS Fellows to WT. WT is supported by JSPS Research Fellowships for Young Scientists.

AUTHOR CONTRIBUTIONS

WT, TT, JAD and SH helped design the study. WT performed most of the experiments, and wrote the first draft. MK measured the enzyme activity. KH measured the ion levels by ion chromatography and by atomic-absorption spectrophotometer. JDB and HT cultivated and provided elephant fish and catshark embryos, respectively. All authors contributed to the analyses and interpretation of the data.

COMPETING INTERESTS

No competing interests declared.

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

661 **Figure 2.** Developmental changes in total mRNA abundance (nmol/fish or
662 YSM) of CPSIII (A), OTC (B), GS1 (C), GS2 (D), and ARG2 (E) in whole embryo
663 and the YSM. Data are presented as means ± SEM. $N = 4$ (developmental stage 28);
664 3 (stage 29); 3 (stage 30); 6 (from stage 31 to 34). Asterisks indicate significant
665 differences (*$P < 0.05$; **$P < 0.01$, ***$P < 0.001$) in the mRNA abundance between
666 whole embryo and YSM in each stage.

667 **Figure 3.** Developmental changes in relative mRNA levels of CPSIII (A),
668 OTC (B), GS1 (C), GS2 (D), and ARG2 (E) in tissues of embryos. Values of each
669 mRNA were normalized against the value of EF1α mRNA as an internal control gene.
670 Developmental increase in embryonic liver weight is shown (F). All data are
671 presented as means ± SEM. $N = 6$ for all samples (stage 33 to hatched fish). H (Bra),
672 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 4. 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 Figure 3. 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 5. (A) Expression patterns and developmental changes of cm*gs* transcripts (GS1Long, GS1Short and GS2) analyzed by RT-PCR. The 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 were also shown. (B) Expression patterns of GS1Long transcript by quantitative PCR. Within each compartment, 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. YSM, yolk sac membrane; H, head; B, body; T, tail; Liv, liver; St., developmental stage.

Figure 6. Morphological observations of the YSM shown by HE staining (A) and localization of CPSIII mRNA by *in situ* hybridization (B, C). (D) Localization of catshark (*Scyliorhinus torazame*) CPSIII mRNA confirmed the

695 expression of CPSIII mRNA in cells of the vascularized endodermal layer of the YSM.
696 Black arrows indicate positive signals of CPSIII mRNA of elephant fish (B) and
697 CPSIII mRNA of catshark (D) transcripts, respectively. ECT, ectoderm; FCL, fibrous
698 connective layer; END, endoderm; BC, blood cell; YPL, yolk platelets. Scale bars,
699 50 μm.
700

Table 1. Composition of egg capsule fluid

Egg capsule fluid	*N*	Osmolality (mOsm kg^{-1})	Na^+ (mmol l^{-1})	Cl^- (mEq l^{-1})	Urea (mmol l^{-1})
At stage 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
SW		1054.0	582.3	574.0	ND

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

715

716

717

Table 2. Composition of embryonic body fluid

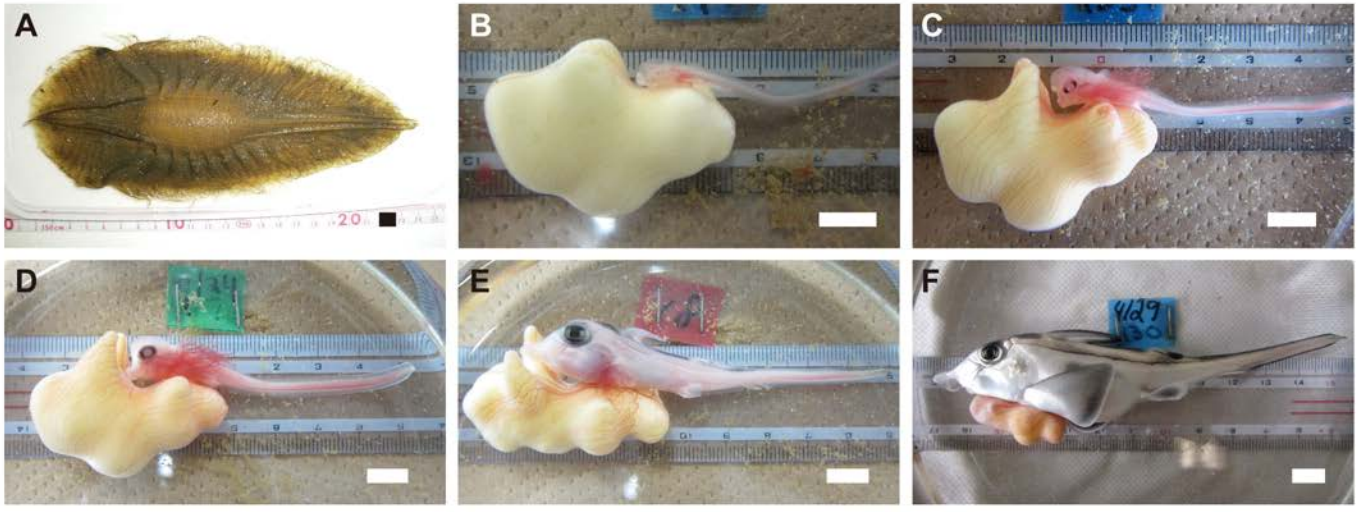
Body fluid	*N*	Osmolality (mOsm kg^{-1})	Na^+ (mmol l^{-1})	Cl^- (mmol l^{-1})	Urea (mmol l^{-1})
At stage 31	1	-	-	-	449
32	6	-	284.5 ± 7.0	290.8 ± 8.0	488.6 ± 13.1
33	7	-	308.4 ± 5.6	307.2 ± 5.4	498.2 ± 8.1
34	3	-	327.3 ± 1.6	295.5 ± 4.3	480.7 ± 7.4
36	12	1048.9 ± 3.1	298.7 ± 4.2	316.3 ± 11.1	473.3 ± 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

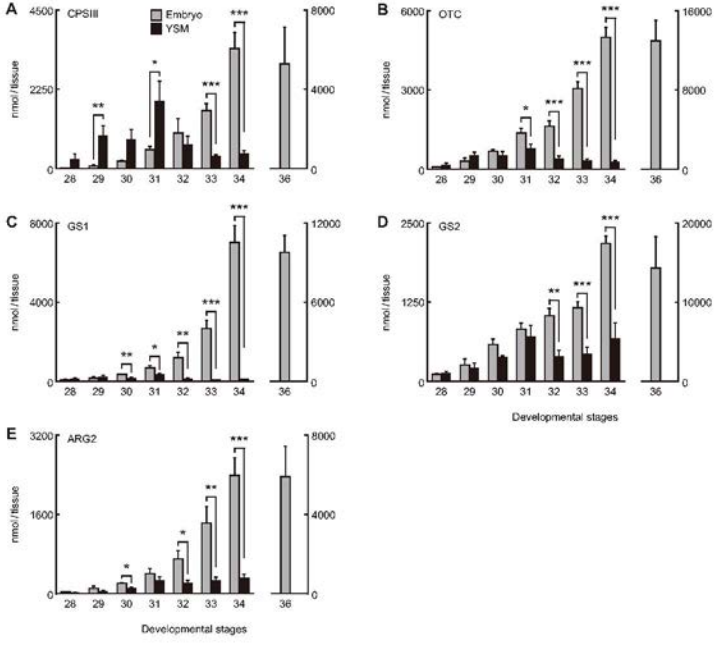
Note. Values are means ± SEM. [1,] Data from Hyodo et al., 2007. Developmental stage 36 in the present study corresponds to pre-hatching ('before hatching') fish referred by Takagi et al. (2012).

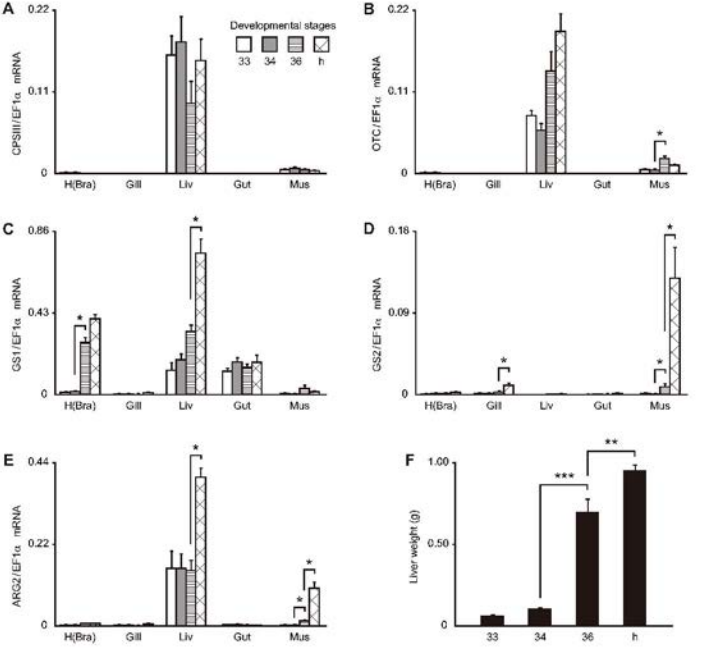
Table 3. Activity of OUC enzymes

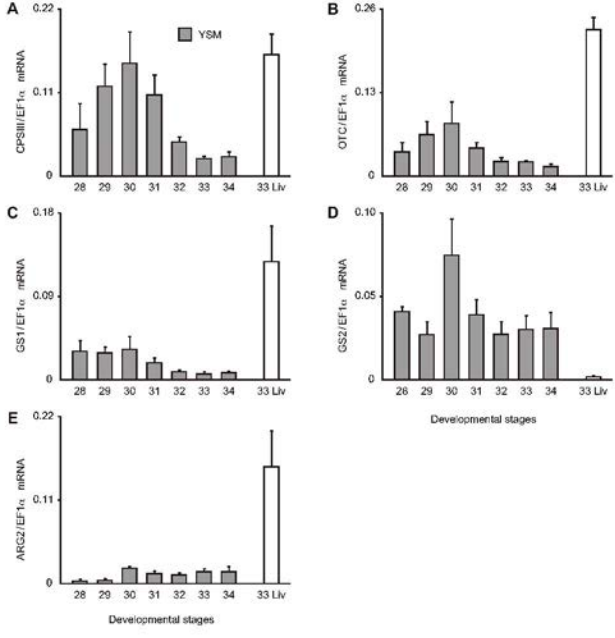
Samples	GS	CPSIII	OTC	ARG
YSM at stage 31	0.43 ± 0.16	0.24 ± 0.04	0.65 ± 0.18	1.17 ± 0.25
YSM at 34	0.34 ± 0.06	0.12 ± 0.04 *	0.73 ± 0.10	1.72 ± 0.19
Liver at 36	15.53 ± 0.92	0.87 ± 0.13	21.88 ± 1.45	23.74 ± 2.26
Adult liver [1]	13.84 ± 1.09	1.07 ± 0.09	31.36 ± 2.12	69.79 ± 4.47

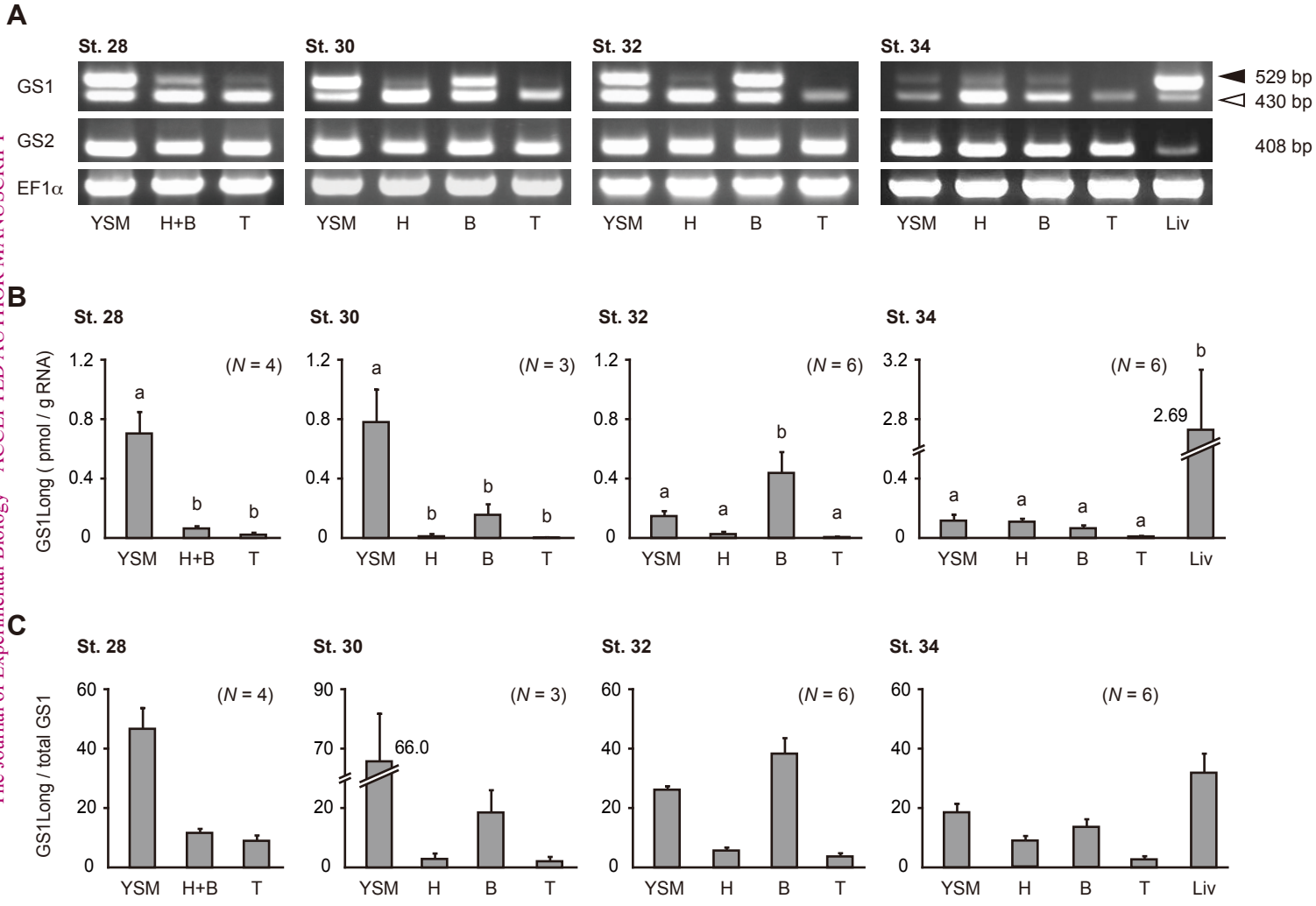
Values for enzyme activities in μmol/min/g were presented as means ± SEM. $N = 5$ (YSM at stage 31); 5 (YSM at 34); 6 (embryonic liver at 36). *, significantly different in CPSIII activities of YSM between stages 31 and 34 at $P < 0.05$. [1]Data of adult fish liver were from Takagi et al., 2012.

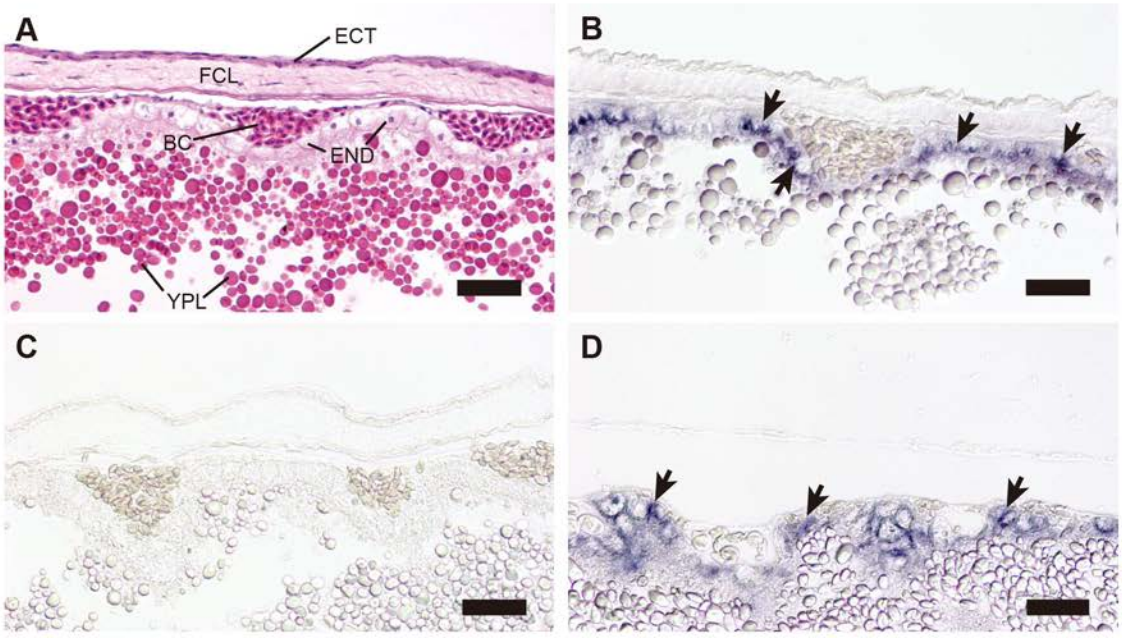

A
B
C
D
E
F


A
CPSIII
Embryo
YSM
nmol/tissue
4500
2250
0
8000
4000
0
28 29 30 31 32 33 34 36
B
OTC
6000
3000
16000
8000
C
GS1
12000
6000
D
GS2
2500
1250
20000
10000
Developmental stages
E
ARG2
3200
1600
Developmental stages


A
CPSIII/EF1α mRNA
0.22
0.11
0
Developmental stages
33 34 36 h
H(Bra) Gill Liv Gut Mus
B
OTC/EF1α mRNA
0.22
0.11
0
H(Bra) Gill Liv Gut Mus
C
GS1/EF1α mRNA
0.86
0.43
0
H(Bra) Gill Liv Gut Mus
D
GS2/EF1α mRNA
0.18
0.09
0
H(Bra) Gill Liv Gut Mus
E
ARG2/EF1α mRNA
0.44
0.22
0
H(Bra) Gill Liv Gut Mus
F
Liver weight (g)
1.00
0.50
0
33 34 36 h


A
0.22
0.11
0
YSM
CPSIII/EF1α mRNA
28 29 30 31 32 33 34 33 Liv
B
0.26
0.13
0
OTC/EF1α mRNA
28 29 30 31 32 33 34 33 Liv
C
0.18
0.09
0
GS1/EF1α mRNA
28 29 30 31 32 33 34 33 Liv
D
0.10
0.05
0
GS2/EF1α mRNA
26 29 30 31 32 33 34 33 Liv
Developmental stages
E
0.22
0.11
0
ARG2/EF1α mRNA
28 29 30 31 32 33 34 33 Liv
Developmental stages




A
ECT
FCL
BC
END
YPL
B
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D