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# Regulation of glycogen metabolism in gills and liver of the euryhaline tilapia (*Oreochromis mossambicus*) during acclimation to seawater

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## Summary

**Glucose, which plays a central role in providing energy for metabolism, is primarily stored as glycogen. The synthesis and degradation of glycogen are mainly initialized by glycogen synthase (GS) and glycogen phosphorylase (GP), respectively. The present study aimed to examine the glycogen metabolism in fish liver and gills during acute exposure to seawater. In tilapia (*Oreochromis mossambicus*) gill, GP, GS and glycogen were immunocytochemically colocalized in a specific group of glycogen-rich (GR) cells, which are adjacent to the gill's main ionocytes, mitochondrion-rich (MR) cells. $Na^+/K^+$-ATPase activity in the gills, protein expression and/or activity of GP and GS and the glycogen content of the gills and liver were examined in tilapia after their acute transfer from freshwater (FW) to 25‰ seawater (SW). Gill $Na^+/K^+$-ATPase activity rapidly increased immediately after SW transfer. Glycogen content in both the gills and liver were significantly depleted after SW transfer, but the depletion occurred earlier in gills than in the liver. Gill GP activity and protein expression were upregulated 1–3 h post-transfer and eventually recovered to the normal level as determined in the control group. At the same time, GS protein expression was downregulated. Similar changes in liver GP and GS protein expression were also observed but they occurred later at 6–12 h post-transfer. In conclusion, GR cells are initially stimulated to provide prompt energy for neighboring MR cells that trigger ion-secretion mechanisms. Several hours later, the liver begins to degrade its glycogen stores for the subsequent energy supply.**

Key words: glycogen phosphorylase, glycogen synthase, $Na^+/K^+$-ATPase, osmoregulation, mitochondrion-rich cells, salinity.

## Introduction

Glucose plays a central role in providing energy for metabolism. It is primarily stored in animal tissues as a long-branching high-molecular-mass polysaccharide called glycogen (Roach et al., 1998). Glycogen metabolism is the principal energy source in both vertebrates and invertebrates, especially during environmental fluctuations (Karlsson, 1979; Hoffman and Katz, 1998; Oliveira et al., 2004; Bacca et al., 2005). The synthesis and degradation of glycogen are mainly initiated by glycogen synthase (GS; EC 2.4.1.11) and glycogen phosphorylase (GP; EC 2.4.1.1), respectively. GS, which constitutes an additional mechanism of control over glycogen metabolism, plays a major role in glycogen storage and is also the rate-limiting step in the glycogen synthesis pathway (Fernandez-Novell et al., 1992; Garcia-Rocha et al., 2001). On the other hand, GP is a rate-limiting enzyme that maintains energy support for various tissues. It is composed of two identical subunits that control the breakdown of glycogen to glucose 1-phosphate (Newgard et al., 1989; Frolow and Milligan, 2004; Greenberg et al., 2006).

Because gills are directly exposed to the outer aquatic environment, they have multiple functions including gas exchange, acid–base balance and ionic/osmotic regulation (Evans et al., 2005). In terms of the mechanisms of ionic/osmotic regulation, mitochondrion-rich (MR) cells in gill epithelia are the main sites responsible for active ion transport functions, which are conducted by the operations of various ion transporters and enzymes. These operations are highly energy-consumptive processes (Hirose et al., 2003; Evans et al., 2005).

It has been well documented that acclimation to hypertonic seawater (SW) in euryhaline teleosts involves a timely and sufficient activation of an ion excretion system. This includes the morphological modifications of gill MR cells and stimulation of both the expression and activity of ion transporters (Hwang, 1987; Hwang et al., 1989; Marshall et al., 1999; Lee et al., 2003). These processes require an additional energy supply, reflecting changes in oxygen consumption in fish upon encountering fluctuations in environmental salinity (Boeuf and Payan, 2001). However, monitoring oxygen consumption, which most studies have emphasized, is not a direct approach for determining energy metabolism in a gill cell that conducts ion regulation (Morgan and Iwama, 1991; Morgan and Iwama,

1998). Several studies have addressed the major role that carbohydrate plays in energy metabolism for osmoregulation. Energy may be mainly supplied by the oxidation of glucose and lactate obtained from the circulation as a result of carbohydrate metabolism (Perry and Walsh, 1989; Morgan et al., 1997). Indeed, consumption of the glycogen content of the liver has been reported in rainbow trout (*Oncorhynchus mykiss*) after transfer to SW (Soengas et al., 1991). Hepatic metabolism was found to be related to salinity acclimation in euryhaline fishes (Nakano et al., 1998; Sangiao-Alvarellos et al., 2003).

In earlier studies on teleost gills, glycogen granules/particles were found in MR cells by electron microscopic observations (Philpott and Copeland, 1963); however, this was not further confirmed by molecular evidence. Recently, an antibody against glycogen was used to identify glycogen deposits localized in a group of cells, glycogen-rich (GR) cells, in tilapia gills, and these GR cells express a gill form of GP (Tseng et al., 2007). Moreover, the glycogen content and expression/activity of GP in these cells were affected by environmental salinity (Tseng et al., 2007). These results imply that glycogenesis and glycogenolysis in fish gills may be involved in energy metabolism during acclimation to salinity changes; however, the detailed mechanism is still unclear. No study has clarified the partitioning of energy supplements between the liver and gills in gill energy requirement during acclimation to salinity changes.

In the present study, we attempted to examine glycogen metabolism in fish liver and gills during acute exposure to SW. Mozambique tilapia (*Oreochromis mossambicus*), a euryhaline cichlid, was selected for study due to its ability to adapt to acute fluctuations in salinity. The following experiments were performed: (1) GS was cloned and sequenced from tilapia, and the expression of GS in various tissues, including the gills, was examined; (2) immunocytochemistry was used to localize GP, GS and glycogen in tilapia gill cells; (3) time-course changes in glycogen content in tilapia liver and gills after exposure to SW were examined; (4) time-course changes in GP protein and/or activity levels in tilapia liver and gills after exposure to SW were examined and (5) time-course changes in GS protein level in tilapia liver and gills after exposure to SW were examined.

## Materials and methods

### *Experimental animals*

Tilapia (*Oreochromis mossambicus* Peters), 40–60 g in body mass and 10–14 cm in total length, were obtained from stocks of the Institute of Cellular and Organismic Biology, Academia Sinica, Taipei, Taiwan. The fish were kept in local freshwater (FW) circulating in a tank at 27–28°C under a 14 h:10 h light:dark photoperiod and were fed daily with artificial feed pellets (Fu-So, Taipei, Taiwan). The experimental protocols were approved by the Academia Sinica Institutional Animal Care and Utilization Committee (approval no. RFiZOOHP2006083).

### *Acclimation experiments*

Seawater (SW) was prepared by adding appropriate amounts of artificial sea salt (Taikong, Taipei, Taiwan) into the FW. In exposure experiments, FW tilapia were directly transferred to 25‰ SW and sampled at 1, 3, 6, 12, 24, 48 and 168 h after the transfer. The fish in the acute exposure experiment were not fed. In total, eight fish were sacrificed at each sampling point: four fish for the experimental treatment (FW to SW) and another four fish for the control (FW transferred to FW). All samples were processed at the same time: 09.30–11.30 h in order to normalize the effects of circadian rhythms on physiological metabolism. Sampled gills and liver were immediately placed in ice-cold 1% phosphate-buffered saline (PBS) and were subsequently subjected to the treatments described below.

### *Isolation of epithelial cells from tilapia gills*

Preliminary experiments indicated that blood cells and muscle cells contain considerable amounts of glycogen, GP and GS. Isolation of epithelial cells from gill tissue is necessary in order to exclude the effects of these non-epithelial cells. The isolation method followed a previous paper (Tseng et al., 2007). Gills were carefully rinsed with PBS and then tissue was scraped from the gill filaments and immediately kept in dissociation buffer (0.5 mol $l^{-1}$ EDTA, 500 μl Percoll in PBS) on ice. After rigorous agitation with a stirring bar on ice for 30 min, gill cells were isolated from the tissue by gently filtering the solution through a 100-μm nylon mesh to remove the larger tissue fragments. The filtered cell suspension was poured into a Percoll (Sigma, St Louis, MO, USA) solution (2:1:1 cell suspension:Percoll:PBS) and centrifuged for 10 min at 2000 ***g*** at 4°C. The epithelial cell fraction was collected, washed with PBS and centrifuged. Afterwards, the isolated cells were subjected to western blotting with an anti-tropomyosin (a muscle-specific protein) monoclonal antibody (mAb) (Sigma) to confirm that there was no contamination of muscle cells (data not shown). The isolated epithelial cells were stored at –80°C.

### *Preparation of mRNA*

In the process of preparing mRNA, 200–300 mg of gill tissues was homogenized in 3 ml of Trizol reagent (Invitrogen, Carlsbad, CA, USA) and treated according to the manufacturer's protocols. The amount and quality of total RNA were determined by measuring the absorbance at 260 and 280 nm with a spectrophotometer (Hitachi U-2000, Tokyo, Japan) and analysis using RNA-denatured gels. The total amount of RNA was subsequently extracted with a QuickPrep Micro mRNA Purification Kit (Amersham Pharmacia, Piscataway, NJ, USA). Finally, the pellets of mRNA were precipitated with 0.1 mg glycogen, 1/10 volume of 3 mol $l^{-1}$ NaOAc and 95% ethanol and stored at –20°C.

### *cDNA synthesis from mRNA*

For cDNA synthesis, 0.36 μg of mRNA was reverse-transcribed in a final volume of 20 μl containing 0.5 mmol $l^{-1}$ dNTPs, 2.5 μmol $l^{-1}$ oligo $(dT)_{18}$, 5 mmol $l^{-1}$ dithiothreitol and 200 units of PowerScript reverse transcriptase (Clontech, Palo Alto, CA, USA) for 1.5 h at 42°C, followed by a 15-min incubation period at 70°C. The cDNA samples were finally stored at –20°C.

### *Cloning of tilapia GS cDNA from the gills*

Degenerate oligonucleotide primers for PCR were designed based on the conserved sequences of GS from different vertebrates. The primer set for cloning GS consisted of forward (5′-AATGTGAAGAAGTTCTCKGC-3′) and reverse (5′-

GCAAAGATGGCYCTCTTCAT-3′) sequences. For PCR amplification, 2 μl cDNA was used as a template in a 50 μl final reaction volume containing 0.25 mmol $l^{-1}$ dNTP, 2.5 units EXTaq polymerase (Takara, Shiga, Japan) and 0.2 μmol $l^{-1}$ of each primer. The PCR products were subcloned into the pGEM-T Easy vector (Promega, Madison, WI, USA), and the nucleotide sequence was determined with an ABI 377 DNA Sequencer (ABI, Warrington, UK). Sequences were analyzed with the BLASTx program (NCBI).

The 5′ and 3′ RACE (rapid amplification of cDNA ends) cDNAs were cloned with a commercial kit (SMART RACE cDNA Amplification Kit; Clontech) following the manufacturer's protocols. The specific primers of GS for 5′ and 3′ RACE were 5′-CGGCGAACACAGTTGAGGATGGG-3′ and 5′-ACAGCAGCGACCCCATCCTCAA-3′, respectively. The RACE PCR products were also subcloned into the pGEM-T Easy vector and sequenced. The entire amino acid sequence deduced by the CLUSTAL program (Higgins and Sharp, 1988; Higgins et al., 1996) was used for multiple sequence alignments and analysis of the phylogenetic tree. The dataset was subjected to a distance analysis using the maximum parsimony (MP analysis) method, and 1000 bootstrap replicates of the analysis were carried out with the MEGA program version 2.1.

### *GS mRNA expression in different tissues*

Total RNA samples were extracted from the brain, gills, liver, muscle, intestines, heart, spleen and kidneys in FW tilapia. The samples were subjected to RT-PCR analysis, and β-actin gene expression was utilized as an internal control. The primer set for GS consisted of the forward (5′-TGGGATACTGCTCAGACT-GTGA-3′) and reverse sequences (5′-TGTCCTCCAGCATG-TTGTGAGT-3′) (a 187-bp fragment), and that for the control β-actin consisted of the forward (5′-CGGAATCCACGAAAC-CACCTA-3′) and reverse sequences (5′-ATCTCCTGCATC-CTGTCA-3′) (a 135-bp fragment). Denaturation was performed for 3 min at 95°C, and thereafter for 30 s at 95°C. The annealing time was 30 s at 57°C, and the elongation time was 30 s at 72°C. The reaction was run for 30 cycles. All amplicons were sequenced to confirm that the PCR products were the desired gene fragments.

### *Western blotting*

Both mouse and tilapia tissues (isolated gill epithelial cells and liver tissues) were homogenized with the homogenization solution (100 mmol $l^{-1}$ imidazole-HCl, 5 mmol $l^{-1}$ $Na_2EDTA$, 200 mmol $l^{-1}$ sucrose and 0.1% sodium deoxycholate; pH 7.6) at 600 revs $min^{-1}$ on ice. After centrifugation at 4°C and 9800 ***g*** for 30 min, the total protein concentration of the supernatant was measured with a protein assay kit (protein assay dye reagent concentrate kit; Bio-Rad, Hercules, CA, USA), and the supernatant was then stored at –80°C. The protein sample (50 μg) was supplemented with 6× electrophoresis sample buffer (250 mmol $l^{-1}$ Tris-base, 2 mmol $l^{-1}$ $Na_2EDTA$, 2% SDS and 5% dithiothreitol) and then incubated at 95°C for 10 min. The denatured samples were subjected to SDS–polyacrylamide gel electrophoresis (SDS-PAGE) at 110 V for 2 h. After being transferred to polyvinylidene difluoride membranes, the blots were incubated in 5% nonfat milk for 3 h at room temperature and then washed twice with PBST buffer (0.01 mol $l^{-1}$ phosphate, 0.09% NaCl, pH 7.5 and 0.05% Tween 20). The blotted membranes were incubated overnight with a rabbit anti-human GS polyclonal antibody (Rockland, Gilbertsville, PA, USA; diluted 1:750) at 4°C. After being washed twice with PBST buffer, the blotted membranes were reacted for another 2 h with an alkaline-phosphatase-conjugated goat anti-rabbit IgG antibody (Jackson ImmunoResearch, Cambridgeshire, UK, diluted 1:1000). After washing with PBST buffer, immunoreactive proteins were visualized with nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in staining buffer. Immunoblots were scanned and exported as JPEG files, and the differences between the band intensities of FW and SW samples were compared using a commercial software package (Image-Pro Plus 4.0; Media Cybernetics, Silver Spring, MD, USA).

### *$Na^+/K^+$-ATPase activity assay*

$Na^+/K^+$-ATPase activity was determined as described by Hwang et al. (Hwang et al., 1989). Isolated gill epithelial cells were briefly homogenized in the homogenization solution [100 mmol $l^{-1}$ imidazole-HCl buffer (pH 7.6), 5 mmol $l^{-1}$ $Na_2EDTA$, 200 mmol $l^{-1}$ sucrose and 0.1% sodium deoxycholate]. The $Na^+/K^+$-ATPase activity was assayed in the reaction mixture containing 100 mmol $l^{-1}$ imidazole-HCl buffer (pH 7.6), 125 mmol $l^{-1}$ NaCl, 75 mmol $l^{-1}$ KCl, 7.5 mmol $l^{-1}$ $MgCl_2$ and 5 mmol $l^{-1}$ $Na_2ATP$. The reaction was run at 37°C for 30 min and then stopped by adding 200 ml of ice-cold 30% trichloroacetic acid. The enzyme activity was defined as the difference between the inorganic phosphates liberated in the presence and absence of 3.75 mmol $l^{-1}$ ouabain in the reaction mixture. Each sample was assayed in triplicate.

### *GP activity assay*

Measurement of gill glycogen phosphorylase activity followed a procedure described by Milligan (Milligan, 2003), with some modifications. Isolated gill epithelial cells were homogenized in ice-cold homogenization solution (100 mmol $l^{-1}$ imidazole, 100 mmol $l^{-1}$ KF, 5 mmol $l^{-1}$ EDTA and 1 mmol $l^{-1}$ phenylmethylsulfonylfluoride). Total GP (GPa+GPb) activity was measured by incubating the samples at 25°C in the presence of 1.6 mmol $l^{-1}$ 5′AMP, 45 mmol $l^{-1}$ potassium-phosphate buffer (pH 7.0), 0.2 mg $ml^{-1}$ glycogen, 0.34 mmol $l^{-1}$ NADP, 4 μmol $l^{-1}$ glucose-1,6-bisphosphate, 0.1 mmol $l^{-1}$ EDTA, 15 mmol $l^{-1}$ $MgCl_2$, 1.6 U $ml^{-1}$ phospho-glucomutase and 12 U $ml^{-1}$ glucose-6-phosphate dehydro-genase. The absorbance changes between the reactions with and without glycogen (the substrate) were measured at 340 nm with a Hitachi U-2000 spectrophotometer (Tokyo, Japan). Each sample was assayed in triplicate.

### *Glycogen content*

Isolated gill epithelial cells and liver tissues were homogenized in 30% KOH and heated to 100°C for 30 min. The samples were supplemented with two volumes of 100% ethanol and incubated overnight. Glycogen was precipitated by centrifugation after the addition of 2–3 drops of $Na_2SO_4$. The glycogen pellets were washed with 66% ethanol and then completely dried. The glycogen content was analyzed in 0.2% anthrone reagent dissolved in $H_2SO_4$ using a Hitachi spectrophotometer.

*Immunocytochemistry*

Fresh gills were fixed with 4% paraformaldehyde at 4°C for 3 h and then gradually immersed in PBS containing 5%, 10% and 20% concentrations of sucrose for 15 min, with each step at room temperature. Finally, they were soaked in a mixed PBS solution (OCT compound:20% sucrose, 1:2) overnight and then embedded in OCT compound embedding medium (Sakura, Tokyo, Japan) at –20°C. Cryosections (5 μm thick) were made with a cryostat (CM 1900; Leica, Heidelberg, Germany) and placed onto poly-L-lysine-coated slides (Erie, Hooksett, NH, USA). Prepared slides were then rinsed in PBS and blocked with 3% bovine serum albumin (BSA) for 30 min. Afterwards, the slides were incubated with the $Na^+/K^+$-ATPase α5 mAb (Hybridoma Bank, University of Iowa, Ames, IA, USA; diluted 1:200), mouse anti-human GP brain form mAb (Biotrend Chemikalien, Cologne, Germany; diluted 1:200) and/or glycogen mAb (Baba, 1993) (diluted 1:200) overnight at 4°C, respectively. The slides were then washed twice with PBS and incubated with anti-mouse IgG conjugated with Texas Red (Jackson ImmunoResearch Lab., West Grove, PA, USA; diluted 1:200) for 2 h at room temperature. After being washed with PBS twice, the slides were incubated again with the anti-human GS polyclonal antibody (diluted 1:200) overnight at 4°C and then washed again before being incubated with anti-rabbit IgG conjugated with fluorescein isothiocyanate (FITC) for 2 h at room temperature. The images were acquired with a Leica TCS-NT confocal laser scanning microscope (Leica Lasertechnik, Heidelberg, Germany). Observations were made in the trailing edge of gills where most of the MR cells locate.

*Statistical analysis*

Values are presented as means ± s.d. ($N$=6). All time-course data were statistically analyzed by two-way analysis of variance (ANOVA), and pairwise multiple comparisons were made by Tukey's test ($P$<0.05).

## Results

*Tilapia glycogen synthase (tGS) cloning and sequencing*

Full-length GS cDNA was cloned and sequenced from tilapia gills (Fig. 1) (GenBank Accession No. EF565371). The complete sequence obtained was 2660 nucleotides (nt) long and contained a 2133-nt open reading frame, a 359-nt 5′-untranslated region and a 168-nt 3′-untranslated region with a poly (A) tail. The deduced amino acid sequence encodes a protein of 711 amino acids. Using commercial software (Vector NTI; Invitrogen, San Diego, CA, USA) to analyze the amino acid sequence, we discovered that the GS from tilapia gills (tGS) is about 70% identical to the zebrafish GS muscle form, 65% to the mouse GS muscle form, 62% to the mouse GS brain form, 55% to the mouse liver form and 62.5% to the human GS muscle form (data not shown). Analysis of sequence alignments indicated that the majority of the sequences were highly conserved (Pederson et al., 2000). The $NH_2$ and COOH termini are the most variable regions and are involved in regulating phosphorylation and dephosphorylation. Five important phosphorylation sites in a mammal (rabbit) GS, *viz.* Ser-650, Ser-654, Ser-658, Ser-662 and Ser-666 (Hardy and Roach, 1993), were also found to be conserved in the COOH termini of tilapia GS (Fig. 1).

*Phylogenetic analysis of the tGS amino acid sequence*

In Fig. 2, a phylogenetic tree was generated based on the MP analysis of the full-length amino acid sequences of the GS isoforms (muscle and liver) from eight species (mouse, rat, rabbit, human, monkey, zebrafish, tilapia and *Drosophila*). The tGS cloned from the gill had the highest homology to the muscle form.

*tGS gene expressions in different tissues*

tGS mRNA expressions in different tissues of tilapia were examined by RT-PCR with β-actin as the internal control. Fig. 3 clearly indicates that the PCR products of the 187-bp fragment of tGS were present. The tGS gene was ubiquitously expressed among various tissues, with higher expression levels in the brain, heart and muscle.

*Western blotting of GS*

Tilapia liver and isolated gill cells, and mouse muscle and kidney cells (as a positive control, following the Product Specification Sheet of Rockland) were subjected to western blotting with an anti-human GS polyclonal antibody. The antibody was raised against a synthetic peptide, A-Q-G-Y-R-Y-P-R-P-A-pS-V-P, which was derived from the 631–643 amino acid sequence of the human muscle GS. The 631–643 amino acid sequence of the tGS and mouse muscle GS showed 77% and 92% identities, respectively, to that of the human muscle GS. As shown in Fig. 4, immunoreactive bands with a similar size were found in all of the tissues examined. The size was about 80.9 kDa in mouse kidney, tilapia liver and gill cells, and 83.9 kDa in mouse muscle. Negative experiments with the pre-immune serum showed no bands (data not shown). These results indicate that the antibody recognizes different iosforms of the tGS and, thus, can be used to detect the tGS protein in gills and liver.

*Localization of GS, $Na^+/K^+$-ATPase, GP and glycogen in tilapia gills*

The specificities of anti-GP, anti-glycogen and anti-$Na^+/K^+$-ATPase antibodies have been confirmed in previous studies (Tseng et al., 2007; Hwang et al., 1999). Double immunohistochemical labeling was conducted to localize GS, GP, glycogen and MR cells. Differential interference contrast (DIC) observations showed distinct images of the outline of the afferent edge of a gill filament (Fig. 5A,D,H). FITC signals for GS, and Texas Red signals for $Na^+/K^+$-ATPase, were both found in gill epithelial cells, but they were not colocalized in the same cells (Fig. 5B,C), clearly indicating that GS was localized in a specific type of cells, GR cells (Tseng et al., 2007) (also see below). Based on confocal-microscopic observations from the serial sections in more than 10 individuals, all these GS-expressing GR cells were adjacent to MR cells (Fig. 5B,C). Moreover, GS and GP were colocalized in the same GR cells (Fig. 5E–G), and GS and glycogen were also found in the same type of cells (Fig. 5I–K). As a result, GS, GP and glycogen were all colocalized in the same group of cells, GR cells, which were recently identified because of their abundant deposits of glycogen (Tseng et al., 2007).

*Time-course changes in gill $Na^+/K^+$-ATPase activity after transfer to 25‰ SW*

FW tilapia were transferred to 25‰ SW and FW (as a

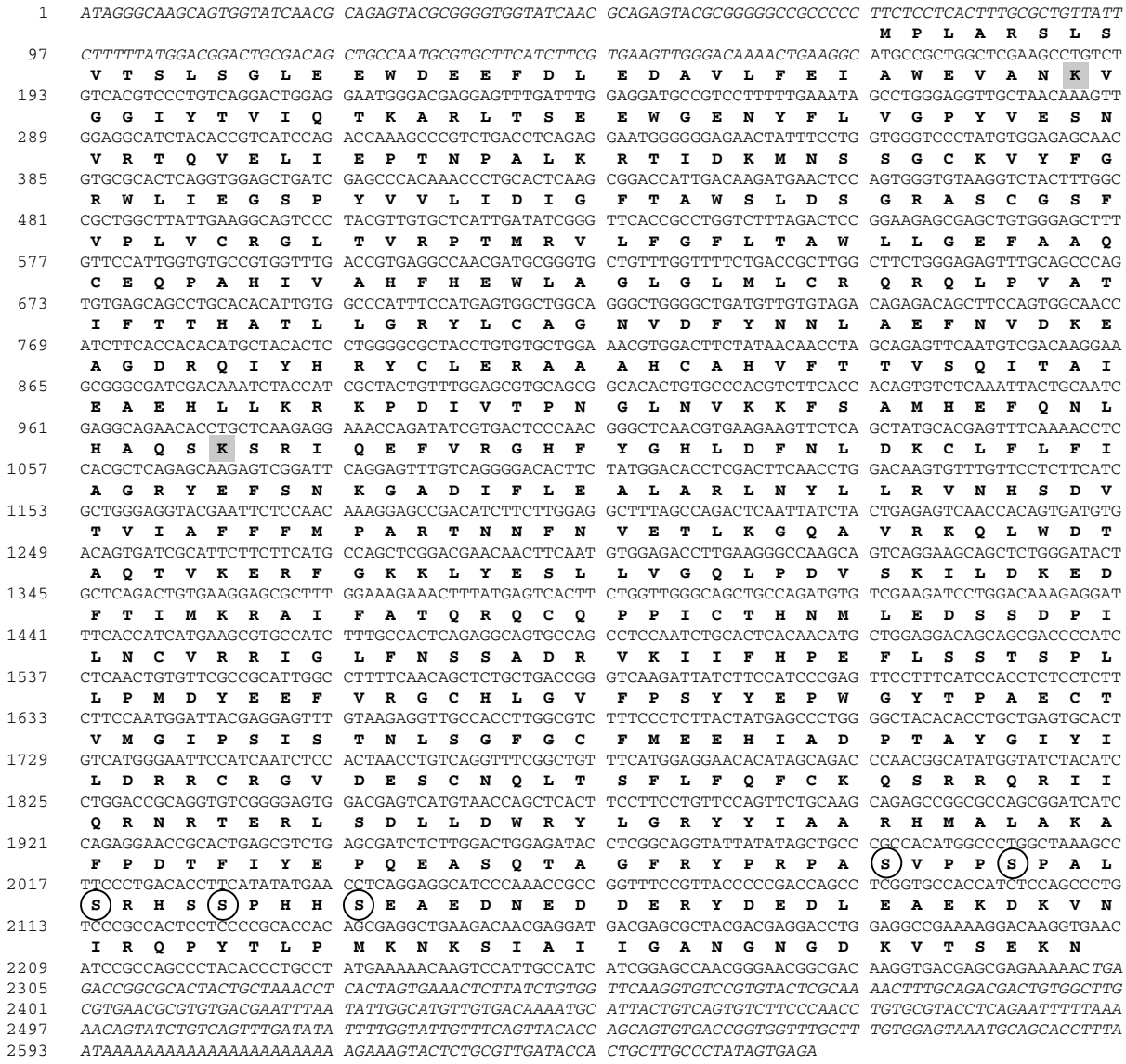

Fig. 1. Full-length glycogen synthase cDNA and the deduced amino acid sequences cloned from tilapia gills. Five important phosphorylation sites (circles) and two UDP-glucose binding-related Lys residues (shaded) are all conserved. GenBank Accession No. EF565371.

control) and then the activity of gill $Na^+/K^+$-ATPase was measured at different time points after the transfer. Gill $Na^+/K^+$-ATPase activity of the FW control group was maintained at 4–6 μmol Pi $h^{-1}$ $mg^{-1}$ protein during the entire experiment (Fig. 6). However, the activity in the 25‰ SW group showed dramatic changes, with two peaks (Fig. 6). The activity exhibited a significant increase (of about 1.9-fold) from the first hour immediately after transfer to 25‰ SW, a high level was maintained during the subsequent 1–6 h, and then the level declined to that of the control. Thereafter, the activity showed another rapid and evident increase 24 h post-transfer, peaked (at about 3.2-fold) again at 48 h, and then maintained a high level until the end of the experiment.

### *Time-course changes in glycogen content in isolated gill and liver cells after transfer to 25‰ SW*

Similar to the above transfer experiment, glycogen content in isolated gill and liver cells was measured at different time points after the transfer. Glycogen content in isolated gill (mainly in GR cells) and liver cells was affected by environmental salinity (Fig. 7A,B). Glycogen deposits in gill GR cells were depleted by about 50% in the first hour immediately after transfer from FW to 25‰ SW, then remained at a low level, and finally recovered to the level of the control (Fig. 7A). On the other hand, it was not until the sixth hour that the glycogen content began to be depleted in the liver, and the content of glycogen in the liver was maintained at a lower level, at ~40–60% of that of the control FW group, until the end of the experiment (Fig. 7B).

### *Time-course changes in GP protein levels and activity, and GS protein levels in isolated gill cells after transfer to 25‰ SW*

Following the changes in the gill $Na^+/K^+$-ATPase activity and glycogen content, gill GP protein levels and activity and GS protein levels also showed dramatic changes (Fig. 8). In isolated gill cells, the patterns of change in the GP protein amount

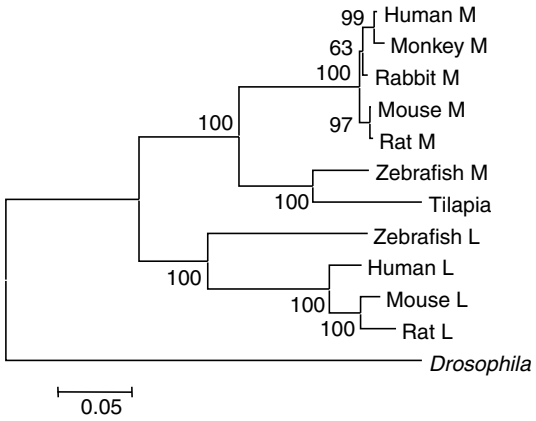


Fig. 2. Phylogenic analysis constructed with complete amino acid sequences of glycogen synthase (GS) by maximum parsimony methods with 1000 replications. The GenBank Accession Nos of the sequences used are as follows: GS muscle form of the mouse, NP_109603; human, NP_002094; rat, XP_341859; rabbit, P13834; monkey, AF529178; zebrafish, NP_957474. GS liver form of the mouse, NP_663547; rat, NP_037221; human, NP_068776; zebrafish, NP_001018199. GS isoform C from *Drosophila*, NP_731968.

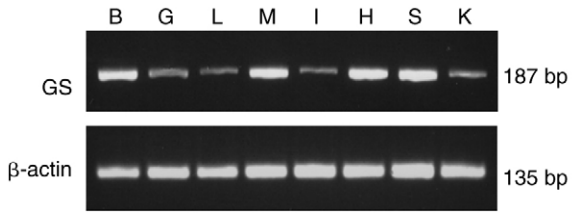


Fig. 3. RT-PCR analysis of glycogen synthase (GS) gene expression in the brain (B), gills (G), liver (L), muscle (M), intestines (I), heart (H), spleen (S) and kidneys (K) of tilapia. β-Actin was used as an internal control. GS was expressed in all of the tissues examined, with the brain, muscle, heart and spleen showing higher expression levels.

(Fig. 8B) and activity (Fig. 8A) were reversed compared with those of the GS protein (Fig. 8C). Gill GP activity began to show evident stimulation (of ~2-fold) from the third hour after transfer to 25‰ SW, it decreased thereafter and then recovered to a stable level similar to that of the control FW group from 12 h post-transfer until the end of the experiment (Fig. 8A). Simultaneously, the gill GP protein amount also increased immediately after transfer, peaked (at ~2-fold) in the third hour and returned to the control level from the 12th hour (Fig. 8B); however, the GP protein amount was higher than that in the control FW group at 168 h at the end of the experiment (Fig. 8B). By contrast, the gill GS protein amount in the SW group showed a sudden decline (to ~60%) in the third hour after transfer, then immediately recovered to the original level thereafter, but it turned out to be higher than the control at the 168th hour at the end of the experiment (Fig. 8C).

### *Time-course changes in GP and GS proteins in the liver after transfer to 25‰ SW*

The profiles of GP and GS protein amounts in the liver were similar to those in isolated gill cells; however, the changes in the liver appeared more slowly than did those in isolated gill cells (Figs 8, 9). As shown in Fig. 9A, the liver GP protein amount appeared to increase from the sixth hour after the transfer to 25‰ SW and returned to the same level as the control group from the 24th hour after transfer (Fig. 9A). By contrast, the liver GS protein amount showed a sudden decrease after 12 h and then immediately returned to the level of the control group (Fig. 9B).

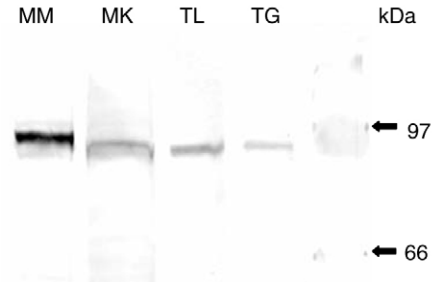


Fig. 4. Western blot analysis of glycogen synthase in mouse muscle (MM), mouse kidney (MK), tilapia liver (TL) and isolated tilapia gill cells (TG). The anti-human glycogen synthase polyclonal antibody revealed immunoreactive bands with similar sizes in all the tissues.

## Discussion

The notable findings of the present study were as follows: (1) GS was cloned and sequenced from fish gills for the first time; (2) GS, GP and glycogen were co-localized in a specific group of gill cells, i.e. GR cells, which are adjacent to MR cells (the major ionocytes); (3) glycogen content in both gill and liver was significantly depleted after acute transfer from FW to 25‰ SW, but the depletion occurred earlier in gills than in the liver; and (4) gill $Na^+/K^+$-ATPase activity rapidly increased immediately after SW transfer. Following this, gill GP activity and protein expression were upregulated 1–3 h post-transfer and eventually recovered, while at the same time GS protein expression was downregulated. Similar changes in liver GP and GS protein expressions were observed but they occurred more slowly, at 6–12 h post-transfer to 25‰ seawater.

The amino acid sequence of the tilapia GS (tGS) from the gills showed the highest identity of 70% with the zebrafish GS muscle form and 55–65% with the mammalian counterparts. Phosphorylation of the C-terminal serine residues of mammalian GS, a key regulator for controlling enzyme activity (Cohen, 1982; Roach, 1990), is conserved in the tGS. Moreover, the tGS also has two Lys residues, Lys38 and Lys300, which have been identified as binding sites for UDP-glucose in the mammalian enzyme (Tagaya et al., 1985; Mahrenholz et al., 1988). Based on these results, the tGS may be a homologue of the mammalian GS muscle form.

The mechanisms of glycogen synthesis and degradation are generally known in mammal tissues, including the liver, muscle and other organs (Smythe and Cohen, 1991; Bollen et al., 1998), and this mechanism seems to be similar in fish gills, an energy-consuming organ. Glycogen was reported to exist in the cytoplasm of fish branchial MR cells according to electron microscopic observations (Philpott and Copeland, 1963; Nakao, 1974). This traditional inference seems unlikely because of the

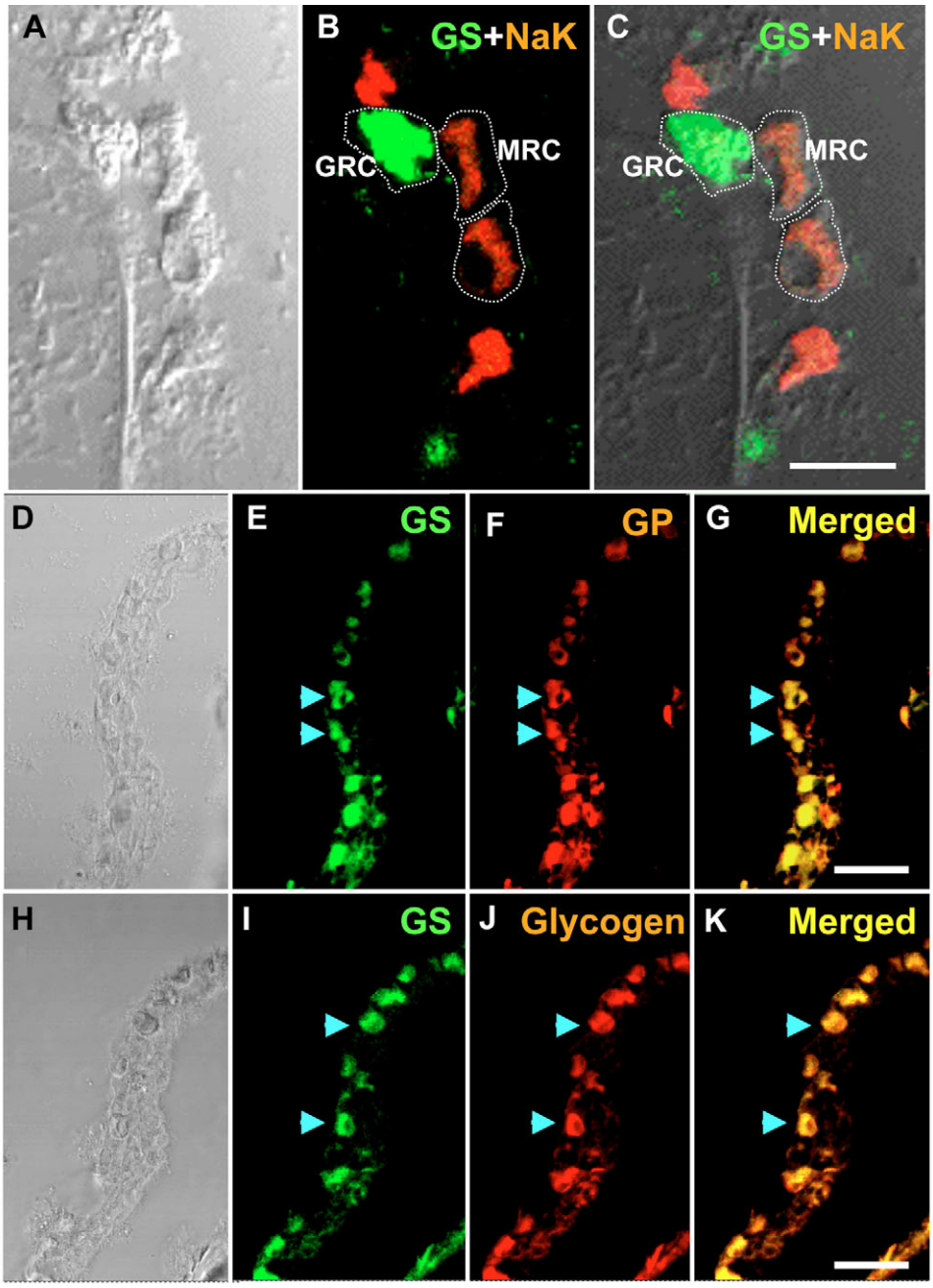


Fig. 5. Immunohistochemical images of freshwater tilapia gill frozen sections. (A–C) Double labeling for glycogen synthase (GS) and $Na^+/K^+$-ATPase (NaK); (D–G) double labeling (arrows) for GS and glycogen phosphorylase (GP); (H–K) double labeling (arrows) for GS and glycogen. A, D and H are DIC images. GS, GP and glycogen were co-localized in a specific group of glycogen-rich cells (GRC) that are adjacent to mitochondrion-rich cells (MRC; labeled with $Na^+/K^+$-ATPase). White dots indicate the outline of the cells. Scale bar: 10 μm (A–C) and 20 μm (D–K).

present and previous (Tseng et al., 2007) convincing molecular and cellular evidence of the colocalization of GP, GS and glycogen in a specific group of cells (GR cells) but not in MR cells in fish gills. As addressed by Tseng et al., GR cells and MR cells share the same apical opening to form a multicellular complex (Tseng et al., 2007). More sensitive analysis, like immuno-electron microscopy, is necessary to further confirm whether GR cells are actually previously reported accessory cells or other transporting cells (Tseng et al., 2007). However, the present result raises the possibility that the relationship in energy translocation between mammal astrocytes and neurons may also occur between GR cells and MR cells in fish gills. In the rat brain, GP and glycogen mainly exist in astrocytes, astroglial cells and ependymal cells of ventricles but never in neurons (Pfeiffer-Guglielmi et al., 2003). During energy deprivation in the central nervous system, glycogen is degraded to lactate, which is shuttled from astrocytes to high-energy-requiring neurons (Ransom and Fern, 1997; Brown et al., 2003; Pfeiffer-Guglielmi et al., 2007).

Acclimation to SW in fish was suggested to be involved in activation of ion secretion pathways in gill MR cells (Evans et al., 2005), and this requires additional energy support (Morgan et al., 1997). Immediately after an acute challenge with SW, gill $Na^+/K^+$-ATPase activity showed a rapid and drastic increase from the first hour in order to recover the impaired internal ion levels and osmolalities in tilapia (Hwang et al., 1989), indicating that a prompt energy supply for stimulating ion-secretion mechanisms is critically needed. Indeed, Morgan et al. (Morgan et al., 1997) examined the oxygen consumption in tilapia after transfer to SW and found a significant increase after 4 days of

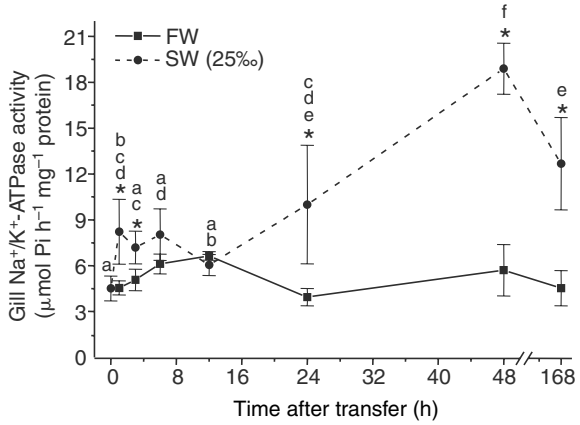


Fig. 6. Time-course changes in gill $Na^+/K^+$-ATPase activities in tilapia transferred from freshwater (FW) to 25‰ seawater (SW). Data are presented as means ± s.d. (*N*=6). *Indicates a significant difference from the respective control in FW ($P$<0.05). Different letters indicate significant differences ($P$<0.05) among sampling times in fish transferred to SW.

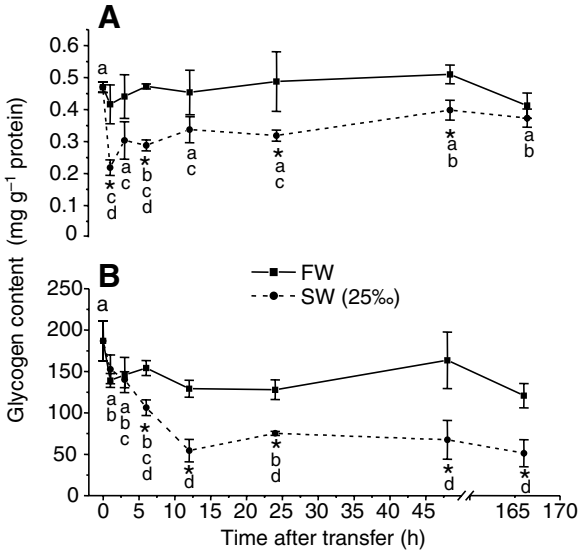


Fig. 7. Time-course changes in gill (A) and liver (B) glycogen content in tilapia transferred from freshwater (FW) to 25‰ seawater (SW). Data are presented as means ± s.d. (*N*=6). *Indicates a significant difference from the respective control in FW ($P$<0.05). Different letters indicate significant differences ($P$<0.05) among sampling times in fish transferred to SW.

acclimation. Other studies, however, reported a reduction in oxygen consumption in tilapia after 2 weeks to 1 month of acclimation to a higher salinity (Febry and Lutz, 1987; Sardella et al., 2004). Most of those previous studies focused on the energetic changes from several days to 1 month. So far, no observations have been made in the case of very short term (in terms of hours) energetic changes, which is critically important for the rapid stimulation of the gill $Na^+/K^+$-ATPase activity observed in the present study.

Carbohydrates, such as glucose and lactate, have been

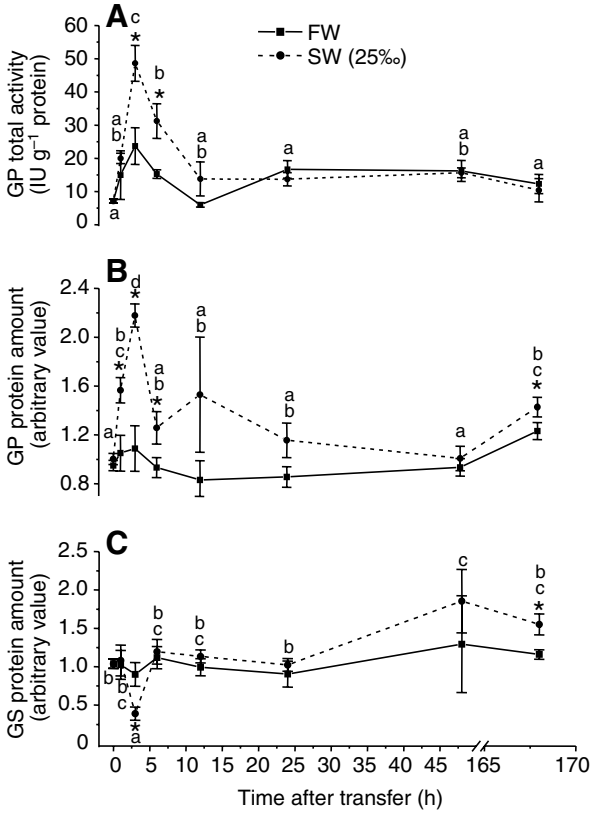


Fig. 8. Time-course changes of gill glycogen phosphorylase (GP) total activity (A), GP protein relative amounts (B) and glycogen synthase (GS) protein relative amounts (C) in tilapia transferred from freshwater (FW) to 25‰ seawater (SW). Protein amounts were measured by western blotting. Data are presented as means ± s.d. (*N*=6). *Indicates a significant difference from the respective control in FW ($P$<0.05). Different letters indicate significant differences ($P$<0.05) among sampling times in fish transferred to SW.

suggested to be an essential energy source for fish osmoregulation during acclimation to different salinities; hence, changes in glycogen content are another suitable indicator for monitoring energetic metabolism in a specific organ or cell (Assem and Hanke, 1979; Nakano et al., 1998; Sangiao-Alvarellos et al., 2005). Effects of environmental salinities on glycogen content in the liver, gills and/or other organs have been examined in several studies. Nakano et al. found no significant changes in liver glycogen contents in tilapia (*O. mossambicus*) after transfer from FW to 23‰ SW for 0.5–96 h (Nakano et al., 1998). Likewise, Assem and Hanke found no significant difference in glycogen content of the liver in the same species 6–168 h after transfer from FW to 25‰ SW (Assem and Hanke, 1979). The controversy between these previous data and the present results in tilapia may be partially due to differences in the fish size, the conditions (e.g. temperature) in which the fish were kept, the method of sampling, etc. On the other hand, Sangiao-Alvarellos et al. examined metabolic changes in the liver and gills in gilthead sea bream (*Sparus auratus*) during acclimation between 38‰ and 55‰ SW and found that glycogen was depleted in the liver on the first day while that in the gills accumulated (Sangiao-Alvarellos et al., 2005). These results imply that mobilization of liver glycogen may provide

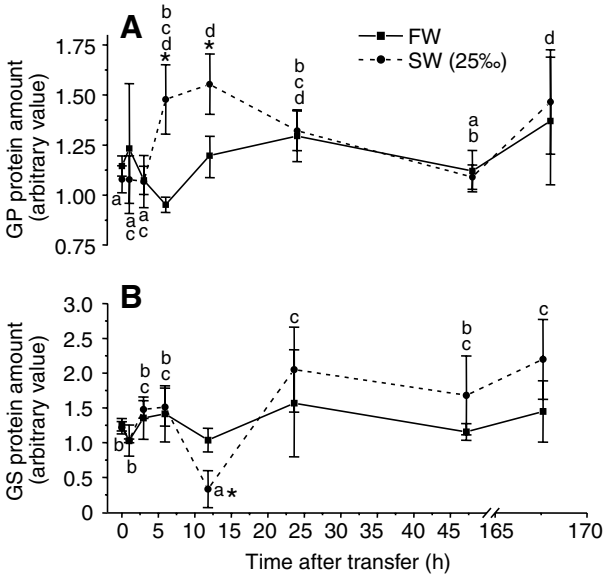


Fig. 9. Time-course changes in liver glycogen phosphorylase (GP) protein relative amounts (A) and glycogen synthase (GS) protein relative amounts (B) in tilapia transferred from freshwater (FW) to 25‰ seawater (SW). Protein amounts were measured by western blotting. Data are presented as means ± s.d. ($N$=6). *Indicates a significant difference from the respective control in FW ($P$<0.05). Different letters indicate significant differences ($P$<0.05) among sampling times in fish transferred to SW.

endogenous carbohydrate fuel to the gills (Sangiao-Alvarellos et al., 2005); however, no further investigations have been done within the first day after transfer, which is a very critical period for a fish exposed to a salinity stress as we addressed above. The present study examines the differential roles of the gills and liver and their spatial and temporal relationships to the energy supply for osmoregulation during acute exposure to a high-salinity environment. The present data of changes in glycogen content indicate that the glycogen in gills may provide prompt energy from the first hour after SW transfer; subsequently, from 6 h post-transfer, liver glycogen may become the major carbohydrate reserve supporting the operation of ion-secretion mechanisms in tilapia gills. Furthermore, the glycogen content in the liver is much greater, about 250–300-fold, than that in the gills, and this also reasonably reflects the different partitions of the two organs in energy supply. SW acclimation stimulates the activities of $Na^+/K^+$-ATPase and other transporters or enzymes in gills, intestine and other osmoregulatory organs (Kelly et al., 1999; Ando et al., 2003; Evans et al., 2005). Liver glycogen may provide energy for all the processes in these organs. It is impossible to distinguish the partitions of liver glycogen to different organs or different transporters; therefore, it is not surprising to find that the profile of liver glycogen (started to deplete at 6 h) was not totally in concert with that of gill $Na^+/K^+$-ATPase (second increase from 24 h).

In both the gills and liver of gilthead sea bream, GP activities and glycogen content changed in parallel immediately after transfer from 38‰ to 55‰ SW, but neither GS activity nor protein expression was studied (Sangiao-Alvarellos et al., 2005). Obviously, these previous findings might not correctly reflect the functional regulation of glycogenesis and glycogenolysis during hyperosmotic acclimation. On the contrary, in the present study, tilapia gill GP and GS protein expressions and/or activities showed opposite changes, increasing (upregulation of glycogenolysis) and decreasing (downregulation of glycogenesis), respectively, from the first to the third hours after transfer to 25‰ SW, and these profiles reasonably reflect depletion of gill glycogen. Similar results were also found in tilapia liver, but stimulation of glycogenolysis in the liver began later, from the sixth to the 12th hour after transfer. In previous studies on rainbow trout (*O. mykiss*), acclimation to 12‰ or 28‰ SW also stimulated liver glycogenolysis, which was associated with an increase in GP activity, a decrease in GS activity and a decline in glycogen levels (Soengas et al., 1991; Soengas et al., 1993). Based on our data, stimulation of glucogenolysis in the gills and thereafter in the liver probably provides a sequential energy supply for the operation of ion-secretion mechanisms, including $Na^+/K^+$-ATPase and other transporters, in tilapia gills during acute exposure to SW.

Glycogen is stored in the liver as a reserve of glucose for extrahepatic tissues, and the liver, acting as a sensor of blood glucose stores, mobilizes glycogen according to peripheral needs (Bollen et al., 1998). Therefore, it is not surprising to find in the present study and other previous work that mobilization of liver glycogen may provide carbohydrate reserves to fuel glycolysis in gills for operation of ion-regulation mechanisms during acclimation to salinity changes (Assem and Hanke, 1979; Soengas et al., 1991; Soengas et al., 1993; Sangiao-Alvarellos et al., 2005). The notable new finding from the present study is that the gills accumulate glycogen as a local carbohydrate reserve, and upon acute salinity stress, the glycogen in the gills is initially degraded to provide a prompt emergency energy supply for activation of salt secretion mechanisms before glycogenolysis in the liver is stimulated. However, direct evidence to demonstrate the energy shuttle between gill cells is still lacking and requires further studies. The role of the gills themselves in the emergency energy supply for osmoregulation seems to have been overlooked in previous studies that examined metabolic changes in gills of the gilthead sea bream during acclimation to different salinities (Sangiao-Alvarellos et al., 2003; Sangiao-Alvarellos et al., 2005). There are some possible reasons for this: first, those studies did not examine short-term changes (in terms of hours) as discussed above; second, perhaps there are differences in the methodologies for measuring glycogen content and GP expression and activity. Our preliminary experiments showed that blood in gill tissues comprised as much as >60% and >80%, respectively, of the glycogen content and GP protein amount in the whole gill (data not shown). Thus, the present study using isolated gill epithelial cells to examine metabolic changes in gill cells avoided the contaminating effects from blood and other cells. The actual changes in gill cells might have been obscured in previous studies by Sangiao-Alvarellos et al. (Sangiao-Alvarellos et al., 2003; Sangiao-Alvarellos et al., 2005), since they used whole gill tissues without isolating the gill cells.

Taking all this into account, we propose a model for carbohydrate metabolism for osmoregulation in tilapia gills during acute exposure to salinity stress: GR cells accumulate

glycogen as a local carbohydrate reserve in gills, glucogenolysis in gill GR cells is initially stimulated to provide prompt energy for neighboring MR cells in order to trigger ion-secretion mechanisms, and several hours later, the liver begins to degrade its glycogen for the subsequent energy supply. This local and systematic partitioning of glycogen metabolism for emergency energy requirements is similar to what has been well documented in mammal brains (neurons and astrocytes) and liver.

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## References

**Ando, M., Mukuda, T. and Kozaka, T.** (2003). Water metabolism in the eel acclimated to sea water: from mouth to intestine. *Comp. Biochem. Physiol.* **136B**, 621-633.

**Assem, H. and Hanke, W.** (1979). Concentrations of carbohydrates during osmotic adjustment of the euryhaline teleost, *Tilapia mossambica*. *Comp. Biochem. Physiol.* **64**, 5-16.

**Baba, O.** (1993). Production of monoclonal antibody that recognizes glycogen and its application for immunohistochemistry. *Kokubyo Gakkai Zasshi* **60**, 264-287.

**Bacca, H., Huvet, A., Fabioux, C., Daniel, J. Y., Delaporte, M., Pouvreau, S., Van Wormhoudt, A. and Moal, J.** (2005). Molecular cloning and seasonal expression of oyster glycogen phosphorylase and glycogen synthase genes. *Comp. Biochem. Physiol.* **140B**, 635-646.

**Boeuf, G. and Payan, P.** (2001). How should salinity influence fish growth? *Comp. Biochem. Physiol.* **130C**, 411-423.

**Bollen, M., Keppens, S. and Stalmans, W.** (1998). Specific features of glycogen metabolism in the liver. *Biochem. J.* **336**, 19-31.

**Brown, A. M., Tekkok, S. B. and Ransom, B. R.** (2003). Glycogen regulation and functional role in mouse white matter. *J. Physiol.* **549**, 501-512.

**Cohen, P.** (1982). The role of protein phosphorylation in neural and hormonal control of cellular activity. *Nature* **296**, 613-620.

**Evans, D. H., Piermarini, P. M. and Choe, K. P.** (2005). The multifunctional fish gill: dominant site of gas exchange, osmoregulation, acid–base regulation, and excretion of nitrogenous waste. *Physiol. Rev.* **85**, 97-177.

**Febry, R. and Lutz, P.** (1987). Energy partitioning in fish: the activity-related cost of osmoregulation in a euryhaline cichlid. *J. Exp. Biol.* **128**, 63-85.

**Fernandez-Novell, J. M., Arino, J., Vilaro, S. and Guinovart, J. J.** (1992). Glucose induces the translocation and the aggregation of glycogen synthase in rat hepatocytes. *Biochem. J.* **281**, 443-448.

**Frolow, J. and Milligan, C. L.** (2004). Hormonal regulation of glycogen metabolism in white muscle slices from rainbow trout (*Oncorhynchus mykiss* Walbaum). *Am. J. Physiol.* **287**, R1344-R1353.

**Garcia-Rocha, M., Roca, A., De La Iglesia, N., Baba, O., Fernandez-Novell, J. M., Ferrer, J. C. and Guinovart, J. J.** (2001). Intracellular distribution of glycogen synthase and glycogen in primary cultured rat hepatocytes. *Biochem. J.* **357**, 17-24.

**Greenberg, C. C., Jurczak, M. J., Danos, A. M. and Brady, M. J.** (2006). Glycogen branches out: new perspectives on the role of glycogen metabolism in the integration of metabolic pathways.*Am. J. Physiol.* **291**, E1-E8.

**Hardy, T. A. and Roach, P. J.** (1993). Control of yeast glycogen synthase-2 by COOH-terminal phosphorylation. *J. Biol. Chem.* **268**, 23799-23805.

**Higgins, D. G. and Sharp, P. M.** (1988). CLUSTAL: a package for performing multiple sequence alignment on a microcomputer. *Gene* **73**, 237-244.

**Higgins, D. G., Thompson, J. D. and Gibson, T. J.** (1996). Using CLUSTAL for multiple sequence alignments. *Meth. Enzymol.* **266**, 383-402.

**Hirose, S., Kaneko, T., Naito, N. and Takei, Y.** (2003). Molecular biology of major components of chloride cells. *Comp. Biochem. Physiol.* **136B**, 593-620.

**Hoffman, J. and Katz, U.** (1998). Glyconeogenesis and urea synthesis in the toad *Bufo viridis* during acclimation to water restriction. *Physiol. Zool.* **71**, 85-92.

**Hwang, P. P.** (1987). Tolerance and ultrastructural response of branchial chloride cells on salinity changes in euryhaline teleost, *Oreochromis mossambicus*. *Mar. Biol.* **94**, 643-649.

**Hwang, P. P., Sun, C. M. and Wu, S. M.** (1989). Changes of plasma osmolality, chloride concentration, and gill Na,K-ATPase activity in tilapia, *Oreochromis mossambicus*, during seawater acclimation. *Mar. Biol.* **100**, 295-299.

**Hwang, P. P., Lee, T. H., Weng, C. F., Fang, M. J. and Cho, G. Y.** (1999). Presence of Na-K-ATPase in mitochondria-rich cells in yolk-sac epithelium of larvae of the teleost, *Oreochromis mossambicus*. *Physiol. Biochem. Zool.* **72**, 138-144.

**Karlsson, J.** (1979). Some features of glycogen metabolism in human skeletal muscle. *Bibl. Nutr. Dieta* **27**, 121-125.

**Kelly, S. P., Chow, I. N. and Woo, N. Y.** (1999). Haloplasticity of black seabream (*Mylio macrocephalus*): hypersaline to freshwater acclimation. *J. Exp. Zool.* **283**, 226-241.

**Lee, T. H., Feng, S. H., Lin, C. H., Hwang, Y. H., Huang, C. L. and Hwang, P. P.** (2003). Ambient salinity modulates the expression of sodium pumps in branchial mitochondria-rich cells of Mozambique tilapia, *Oreochromis mossambicus*. *Zool. Sci.* **20**, 29-36.

**Mahrenholz, A. M., Wang, Y. H. and Roach, P. J.** (1988). Catalytic site of rabbit glycogen synthase isozymes. Identification of an active site lysine close to the amino terminus of the subunit. *J. Biol. Chem.* **263**, 10561-10567.

**Marshall, W. S., Emberley, T. R., Singer, T. D., Bryson, S. E. and McCormick, S. D.** (1999). Time course of salinity adaptation in a strongly euryhaline estuarine teleost, *Fundulus heteroclitus*: a multivariable approach. *J. Exp. Biol.* **202**, 1535-1544.

**Milligan, C. L.** (2003). A regulatory role for cortisol in muscle glycogen metabolism in rainbow trout *Oncorhynchus mykiss* Walbaum. *J. Exp. Biol.* **206**, 3167-3173.

**Morgan, J. D. and Iwama, G. K.** (1991). Effects of salinity on growth, metabolism, and ion regulation in juvenile rainbow trout (*Oncorhynchus mykiss*) and fall Chinook salmon (*Oncorhynchus tshawytscha*). *Can. J. Fish. Aquat. Sci.* **48**, 2083-2094.

**Morgan, J. D. and Iwama, G. K.** (1998). Salinity effects on oxygen consumption, gill $Na^+$, $K^+$-ATPase and ion regulation in juvenile coho salmon. *J. Fish Biol.* **53**, 1110-1119.

**Morgan, J. D., Sakamoto, T., Grau, E. G. and Iwama, G. K.** (1997). Physiological and respiratory responses of the Mozambique tilapia (*Oreochromis mossambicus*) to salinity acclimation. *Comp. Biochem. Physiol.* **117A**, 391-398.

**Nakano, K., Tagawa, M., Takemura, A. and Hirano, T.** (1998). Temporal changes in liver carbohydrate metabolism associated with seawater transfer in *Oreochromis mossambicus*. *Comp. Biochem. Physiol.* **119B**, 721-728.

**Nakao, T.** (1974). Fine structure of the agranular cytoplasmic tubules in the lamprey chloride cells. *Anat. Rec.* **178**, 49-61.

**Newgard, C. B., Hwang, P. K. and Fletterick, R. J.** (1989). The family of glycogen phosphorylases: structure and function. *Crit. Rev. Biochem. Mol. Biol.* **24**, 69-99.

**Oliveira, G. T., Rossi, I. C., Kucharski, L. C. and Da Silva, R. S.** (2004). Hepatopancreas gluconeogenesis and glycogen content during fasting in crabs previously maintained on a high-protein or carbohydrate-rich diet. *Comp. Biochem. Physiol.* **137A**, 383-390.

**Pederson, B. A., Cheng, C., Wilson, W. A. and Roach, P. J.** (2000). Regulation of glycogen synthase. Identification of residues involved in regulation by the allosteric ligand glucose-6-P and by phosphorylation. *J. Biol. Chem.* **275**, 27753-27761.

**Perry, S. F. and Walsh, P. J.** (1989). Metabolism of isolated fish gill cells: contribution of epithelial chloride cells. *J. Exp. Biol.* **144**, 507-520.

**Pfeiffer-Guglielmi, B., Fleckenstein, B., Jung, G. and Hamprecht, B.** (2003). Immunocytochemical localization of glycogen phosphorylase isozymes in rat nervous tissues by using isozyme-specific antibodies. *J. Neurochem.* **85**, 73-81.

**Pfeiffer-Guglielmi, B., Francke, M., Reichenbach, A. and Hamprecht, B.** (2007). Glycogen phosphorylase isozymes and energy metabolism in the rat peripheral nervous system–an immunocytochemical study. *Brain Res.* **1136**, 20-27.

**Philpott, C. W. and Copeland, D. E.** (1963). Fine structure of chloride cells from three species of *Fundulus*. *J. Cell Biol.* **18**, 389-404.

**Ransom, B. R. and Fern, R.** (1997). Does astrocytic glycogen benefit axonal function and survival in CNS white matter during glucose deprivation? *Glia* **21**, 134-141.

**Roach, P. J.** (1990). Control of glycogen synthase by hierarchal protein phosphorylation. *FASEB J.* **4**, 2961-2968.

**Roach, P. J., Cheng, C., Huang, D., Lin, A., Mu, J., Skurat, A. V., Wilson, W. and Zhai, L.** (1998). Novel aspects of the regulation of glycogen storage. *J. Basic Clin. Physiol. Pharmacol.* **9**, 139-151.

**Sangiao-Alvarellos, S., Laiz-Carrion, R., Guzman, J. M., Martin del Rio,**

**M. P., Miguez, J. M., Mancera, J. M. and Soengas, J. L.** (2003). Acclimation of *Sparus aurata* to various salinities alters energy metabolism of osmoregulatory and nonosmoregulatory organs. *Am. J. Physiol.* **285**, R897-R907.

**Sangiao-Alvarellos, S., Arjona, F. J., Martin del Rio, M. P., Miguez, J. M., Mancera, J. M. and Soengas, J. L.** (2005). Time course of osmoregulatory and metabolic changes during osmotic acclimation in *Sparus auratus*. *J. Exp. Biol.* **208**, 4291-4304.

**Sardella, B. A., Matey, V., Cooper, J., Gonzalez, R. J. and Brauner, C. J.** (2004). Physiological, biochemical and morphological indicators of osmoregulatory stress in 'California' Mozambique tilapia (*Oreochromis mossambicus* × *O. urolepis hornorum*) exposed to hypersaline water. *J. Exp. Biol.* **207**, 1399-1413.

**Smythe, C. and Cohen, P.** (1991). The discovery of glycogenin and the priming mechanism for glycogen biogenesis. *Eur. J. Biochem.* **200**, 625-631.

**Soengas, J. L., Otero, J., Fuentes, J., Andres, M. D. and Aldegunde, M.** (1991). Preliminary studies on carbohydrate metabolism changes in domesticated rainbow trout (*Oncorhynchus mykiss*) transferred to diluted seawater (12 p.p.t.). *Comp. Biochem. Physiol.* **98B**, 53-57.

**Soengas, J. L., Barciela, P., Fuentes, J., Otero, J., Andres, M. D. and Aldegunde, M.** (1993). The effect of seawater transfer in liver carbohydrate metabolism of domesticated rainbow trout (*Oncorhynchus mykiss*). *Comp. Biochem. Physiol.* **105B**, 337-343.

**Tagaya, M., Nakano, K. and Fukui, T.** (1985). A new affinity labeling reagent for the active site of glycogen synthase. Uridine diphosphopyridoxal. *J. Biol. Chem.* **260**, 6670-6676.

**Tseng, Y. C., Huang, C. J., Chang, J. C., Teng, W. Y., Baba, O., Fann, M. J. and Hwang, P. P.** (2007). Glycogen phosphorylase in glycogen-rich cells is involved in the energy supply for ion regulation in fish gill epithelia. *Am. J. Physiol.* **293**, R482-R491.