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Involvement of glucose in freeze tolerance in the Japanese tree frog *Hyla japonica*

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ABSTRACT

Glycerol and aquaporin 9 (aquaglyceroporin) are known to be involved in freeze tolerance in the Japanese tree frog *Hyla japonica*. However, the regulatory mechanisms of freeze tolerance in this species have not been fully elucidated. In the present study, we focused on the inter- and intra-cellular dynamics of glucose to analyze the role of glucose and glucose-related proteins such as transporter and metabolic enzymes in freeze tolerance. Serum glucose concentrations were compared among the frogs that were non-hibernating, hibernating, and thawed after freezing at −4°C for 6 h. Serum concentrations of glucose in thawed frogs were significantly higher than those in hibernating and non-hibernating, active frogs. Periodic acid-Schiff staining showed that the accumulation of glycogen in the hepatocytes increased before hibernation and decreased after freezing and thawing. Subsequently, the mRNA expression levels of type 2 glucose transporter (*glut2*), glucose-6-phosphatase, liver glycogen phosphorylase (*pygl*), and type 2 glycogen synthase (*gys2*) in the liver of active, hibernating, frozen or thawed frogs were analyzed by quantitative PCR. Compared with active frogs, *glut2* expression increased in frozen frogs, *pygl* increased in frozen or thawed frogs, and *gys2* increased in hibernating frogs. Immunopositive signals for Glut2 were distinctly observed on the plasma membrane of hepatocytes in hibernating and frozen frogs, but the signal intensity was low in non-hibernating frogs and frogs thawed after freezing. The results obtained indicate that glucose, together with glycerol, acts as a cryoprotectant in *H. japonica* and that its synthesis and accumulation in the liver are enhanced during hibernation or freezing.

Keywords: freeze tolerance, glucose, glucose transporter, hibernation, Japanese tree frog

INTRODUCTION

Freezing of body fluids causes severe cell damage; therefore, several anuran species have developed freeze tolerance mechanisms to protect cells from repeated freezing and thawing during winter. Many of the freeze-tolerant frogs utilize low-molecular-weight solutes as cryoprotectants (Storey & Storey, 2017). Japanese tree frog (*Hyla japonica*), which is widely distributed in the Japanese archipelago, Sakhalin, and the Eurasian continent, survives cold winter conditions (Petrosyan & Kuzmin, 2016). During winter, this species tolerates freezing of body fluids using glycerol as a cryoprotectant and aquaporin 9–an aquaglyceroporin–as a glycerol transporter, respectively (Hirota et al., 2015). Glycerol is known to play an important role in freeze tolerance in some frogs belonging to the genus *Hyla* (Layne and Jones, 2001) and to function as a cryoprotectant in combination with glucose in most cases (Irwin and Lee, 2003; Layne, 1999; Layne and Stapleton, 2009; Storey and Storey, 1985a, 1986).

Along with glycerol, glucose is one of the key cryoprotectants in anuran species; it reduces water loss, stabilizes cells, and increases the survival rate after freezing (Costanzo & Lee, 2013; Constanzo et al., 1993; Devireddy et al., 1999). In most freeze-tolerant frogs such as *Hyla versicolor* (Storey & Storey, 1985a), *Hyla chrysoscelis* (Costanzo et al., 1992), *Rana sylvatica* (Storey & Storey, 1985b; Storey & Storey, 1984), and *Pseudacris crucifer* (Churchill & Storey, 1996), an increase in the plasma glucose concentration and glucose accumulation in peripheral organs are facilitated when the frogs are exposed to temperatures below the freezing point. Moreover, it has been shown that glucose injection augments survival rate of the frogs under frozen condition, decreasing body ice content, and erythrocyte injury (Constanzo et al., 1993). These results strongly suggest that glucose acts as a cryoprotectant also in *H. japonica*.

In general, glucose production is directly triggered by the initiation of freezing (Storey & Storey, 2017). Cryoprotectant glucose is synthesized from liver glycogen in frogs.

Glycogen synthase is an essential enzyme for glycogenesis, and type 2 glycogen synthase (Gys2), the expression of which is restricted to the liver, contributes to glycogenesis in the liver of most vertebrates. Three metabolic enzymes—liver glycogen phosphorylase (Pygl), phosphoglucomutase, and glucose-6-phosphatase (G6p)—are involved in glycogenolysis and the synthesis of glucose in the liver (Storey & Storey, 2017).

Glucose transport can be mediated by facilitative glucose transporters (Gluts) belonging to the major facilitator superfamily of membrane transporters. In mammals, 14 isoforms of Glut have been classified into three classes based on their sequence similarities (Thorens & Mueckler, 2010). In the mammalian liver, protein or mRNA expression of various Glut isoforms (Class I, Glut1–4; Class II, Glut5, 9, and 11; and Class III, Glut6, 8, 10, and 12) has been detected (Karim et al., 2012). Among them, Glut2 is primarily responsible for glucose transport in the liver. Glut2 processes high sugar concentrations efficiently because of its high Vmax and *K*m for glucose (Leturque et al., 2009).

So far there is no report on the involvement of glucose in *H. japonica*. In the present study, we aimed to elucidate further the mechanism of freeze tolerance in *H. japonica*, and intra- and inter-cellular dynamics of glucose under the freezing condition were particularly focused on. For this purpose, measurement of serum glucose levels and analyses of the mRNA expression of the glucose transporter *glut2* and metabolic enzymes during freezing and thawing were conducted. Immunohistochemical studies on the distribution of Glut2 protein in the liver was also performed.

2. MATERIALS AND METHODS

2.1. Animals

Adult Japanese tree frogs, *H. japonica*, harvested from the fields in the vicinity of Tokyo, Japan, were supplied by Oh-uchi Aquatic Animal Supply (Saitama, Japan) in November 2019. The snout–vent length and weight of the frogs were approximately 3.0 cm

and 2.8 g, respectively. Frogs were kept in plastic containers until they went into hibernation under the natural conditions of temperature and light cycles, and the consequential hibernating frogs were used for the freezing experiment during the period from December to February. They were divided into three groups: hibernating frogs, frozen at −4°C for 6 h, and thawed after freezing. A freezing experiment was performed as described previously (Hirota et al., 2015). Active frogs captured in May were kept for 1 week under normal laboratory conditions before sampling. The frogs in each group were dissected, and the blood and tissue samples collected were stored at −80°C until use. The excised tissues were also fixed in a 4% paraformaldehyde solution for histological analyses. All animal experiments were performed in accordance with the guidelines for the care and use of laboratory animals of Shizuoka University and were approved by the Institutional Animal Care and Use Committees of Shizuoka University.

2.2. Measurement of serum glucose

Serum glucose concentrations were measured using a Glucose CII-test Wako kit (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan) according to the manufacturer's instructions.

2.3. Histological analyses

H. japonica liver fixed in 4% paraformaldehyde was embedded in Paraplast plus (McCormick Scientific, St. Louis, MO, USA) and sectioned at 4 μm thickness. Hematoxylin-eosin (HE) staining was performed using the conventional method. For periodic acid-Schiff (PAS) staining, the sections were pretreated with 1% periodic acid and then stained in a cold Schiff reagent (Muto Pure Chemical, Tokyo, Japan) followed by hematoxylin staining of the nucleus. Amylase digestion in advance of the PAS staining was carried out as follows: the sections were incubated in a 0.1 M phosphate buffer (pH 6.8) containing 10 mg/mL amylase at 37°C for 2 h. For immunohistochemistry of Gult2, the sections were covered with a

purified anti-*H. japonica* Glut2 antibody (1:200, as described below) for 16 h and then reacted with a secondary antibody solution containing Alexa Fluor 488-labeled donkey anti-rabbit IgG (1:200; Jackson Immunoresearch, West Grove, PA, USA) and 4′,6-diamidino-2-phenylindole for 2 h. The specificity of immunohistochemistry was verified using antibody that had been preadsorbed with 10 μg/mL of the antigen peptide.

2.4. Molecular cloning of cDNA encoding Hyla japonica glut2, g6p, pygl, and gys2

The sequences of all PCR primers used in this study are listed in Table 1. Total RNA was extracted from the *H. japonica* liver using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA extracted from the liver (3 μg) was reverse transcribed using M-MLV reverse transcriptase (Invitrogen) and an oligo-deoxythymidine $(dT)_{12–18}$ primer. Fragments of *H. japonica glut2*, *g6p*, *pygl*, and *gys2* cDNA were obtained by reverse transcription (RT)-PCR using specific S1 and A1 primers. PCR amplification was performed with TaKaRa Ex Taq DNA polymerase (Takara Bio, Shiga, Japan). Conditions of the PCR were as follows: 94°C for 1 min followed by 35 cycles of 94°C for 30 s, 40°C for 30 s, and 72°C for 1 min. The purified PCR products were subcloned into the pMD20 T-vector (Takara Bio) and sequenced.

The unknown sequences, including the 5′- and 3′-untranslated regions, of the tree frog *glut2*, *g6p*, *pygl*, and *gys2* were identified by 5′- and 3′-rapid amplification of cDNA ends (RACE). For 5′-RACE, total RNA from the liver was reverse transcribed with each 5′ A1 primer. Poly(A) was added to the 3′ terminal of the single-strand cDNA using terminal deoxynucleotidyl transferase (Takara Bio). Using the poly(A)-tailed single-strand cDNA as a template with adaptor-oligo(dT) primer and each 5′ A2 primer, the first-round PCR was carried out. Nested PCR was performed with the adaptor and 5′ A3 primers, using the diluted first-round PCR reaction solution as a template. The first- and nested-PCR comprised 30 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 2 min. For 3′-RACE, the first-strand cDNA was synthesized using the adaptor-oligo(dT) primer and the first-round PCR was

125 performed with the adaptor primer and each 3′ S1 primer. Using the diluted first-round PCR
126 reaction solution as a template, nested PCR was performed with the adaptor and 3′ S2
127 primers. The first- and nested-PCR comprised 30 cycles of 94°C for 30 s, 50°C for 30 s, and
128 72°C for 2 min. The purified RACE products were subcloned into the pMD20 T-vector
129 (Takara Bio) and sequenced.

130 *2.5. Generation of antibody against Hyla japonica Glut2 and western blotting*

131 A peptide corresponding to the C-terminal amino acid residues of Glut2 (491–504) was
132 synthesized, coupled to keyhole limpet hemocyanin, and used as an antigen for immunization
133 of a rabbit (Eurofins Genomics, Tokyo, Japan). To obtain specific antibody for Glut2, the
134 antiserum was purified using an affinity column of CNBr-activated Sepharose 4B (Cytiva,
135 Tokyo, Japan) coupled with the antigen peptide. To check specificity of the antibody, western
136 blotting was conducted using the fraction containing the plasma membrane of the *H. japonica*
137 liver, prepared according to Rosendale et al. (2014).

138 *2.6. RT-PCR and quantitative PCR*

139 Total RNA extracted from various organs was reverse transcribed as described above.
140 Specific primer pairs (PCRS and PCRA) for *glut2*, *g6p*, *pygl*, *gys2*, and glyceraldehyde-3-
141 phosphate dehydrogenase (*gapdh*) were used in RT-PCR. The PCR reactions were performed
142 under the following conditions: 94°C for 1 min followed by 30 (*glut2*, *g6p*, *pygl*, and *gys2*) or
143 25 (*gapdh*) cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s. The authenticity of the
144 PCR products was validated by sequencing.

145 The quantitative RT-PCR (qPCR) assay was conducted on a LightCycler 480 system
146 (Roche Diagnostics, Mannheim, Germany) with reaction solution containing the liver cDNA,
147 each of the specific primers (PCRS and PCRA), and FastStart Essential DNA Green Master
148 (Roche Diagnostics) in each well of a 384-well plate. The assay was performed in duplicate.
149 The PCR conditions were denaturation at 95°C for 5 min followed by 45 cycles of
150 denaturation (95°C, 10 s), annealing (60°C, 10 s), and extension (72°C, 10 s). To assess the

specificity of PCR amplification, a melting curve analysis was carried out from 65°C to 97°C after the amplification. The expression levels of *glut2*, *g6p*, *pygl*, and *gys2* mRNA were calculated according to a standard curve and normalized to the *gapdh* mRNA levels using the LightCycler 480 multiple plate analysis software (Roche Diagnostics). Total RNA without RT and water were used as negative controls for the qPCR analysis.

2.7. Statistical analysis

Tukey's test was used to assess the values obtained in each experiment. A *p*-value of less than 0.05 was considered significantly different.

3. RESULTS AND DISCUSSION

To determine whether glucose acts as a cryoprotectant in addition to glycerol in *H. japonica*, the serum glucose concentration in active, hibernating, frozen, or thawed frogs was measured. The serum glucose concentration did not differ between active and hibernating frogs, but it was approximately five-fold higher in thawed frogs than in active or hibernating frogs (Figure 1a). In the frozen frog group, the blood remained in the liquid phase, but the intensity and frequency of heartbeat were considerably lower than those in the other three groups. Therefore, it was not feasible to collect a sufficient amount of blood for the measurement of glucose. In *R. sylvatica*, heartbeat speed increases within 1 min after freezing and becomes slower after 1 h after freezing (Layne et al., 1989). In *H. japonica*, as in *R. sylvatica*, it is possible that glucose is distributed to the whole body before the heartbeat reaches an extremely low level. PAS staining showed that the polysaccharide content in the liver was higher in hibernated frogs than in active ones (Figure 1b, c). It was also revealed that the accumulated substances in the liver were decreased in the frozen and thawed groups in comparison with the hibernating group (Figure 1c–e). The PAS-stained substance was confirmed to be glycogen because the staining vanished by amylase digestion (Figure S1). These results suggest that glucose as well as glycerol is utilized as a cryoprotectant in *H.*

japonica and that these small solutes contribute to the regulation of body fluids osmolality and freezing point depression. In addition, these results also suggest that the source of glucose is glycogen accumulated in the liver during autumn. Glucose concentrations in the cells may be higher than that in the blood, because not only accumulation of glucose in the cells but also water transport to the extracellular space occurs during freezing. In addition, cryoprotectant acts in combination with other molecules, such as ice-binding protein and membrane protectant (Storey & Storey, 2017). These additive factors are considered to contribute to the freeze tolerance. ~~It was also shown that the source of glucose and glycerol is glycogen accumulated in the liver during autumn.~~

Using 5′- and 3′-RACE, cDNA encoding *glut2*, *g6p*, *pygl*, and *gys2* were cloned from the *H. japonica* liver. The 1663 bp of *glut2* cDNA included 1515 bp of a coding region, which encoded 504 amino acid residues (the sequence data has been submitted to the DDBJ/EMBL/GenBank databases under accession number LC698650). The amino acid sequence of *H. japonica* Glut2 predicted from the cDNA sequence showed 79%, 62%, 62%, 60%, and 58% identity with that of *Xenopus tropicalis* (NP_001011453; Klein et al., 2002), zebrafish (NP_001036186, XP_694431; Marín-Juez et al., 2015), chicken (NP_997061; Parker et al., 2015), mouse (NP_112474; Chhabra et al., 2016), and green anole lizard (XP_008104242) Glut2 proteins, respectively. The predicted amino acid sequence of Glut2 exhibited the typical features of a glucose transporter: 12 transmembrane domains (TMHMM Server v. 2.0, https://services.healthtech.dtu.dk/service.php?TMHMM-2.0) (Krogh et al., 2001), a QLS motif (QFS for Glut2 of most vertebrates) located in the seventh transmembrane segment, and 45 putative substrate translocation pores.

H. japonica g6p cDNA (LC698648) contained a 1086 bp coding region encoding 361 amino acid residues. The predicted sequence of *H. japonica* G6p, including six putative active sites, showed high similarity to G6p of other vertebrates. For *H. japonica pygl* cDNA (LC698649), a 2610 bp of coding region encoded 869 amino acid residues. The 14th amino

203 acid residue of *H. japonica* Pygl was serine, the phosphorylation of which is important for the
204 activation of this enzyme. *H. japonica* Pygl contained 21 putative active site pockets, which
205 exhibited high sequence identity with those of other vertebrates' Pygl. *H. japonica gys2*
206 cDNA (LC698651) included a 2124 bp coding sequence that encodes 707 amino acid
207 residues, and the amino acid sequence of Gys2 is highly conserved among vertebrates; the
208 amino acid sequence of *H. japonica* Gys2 showed 87%, 84%, 84%, 84%, and 80% identity
209 with that of Gys2 of *X. tropicalis* (NP_001015798), chicken (XP_004938048), green anole
210 lizard (XP_003220810), mouse (NP_663547; Kuma et al., 2004), and medaka
211 (XP_020569762), respectively.

212 RT-PCR was performed to examine the distribution of the *glut2*, *g6p*, *pygl*, and *gys2*
213 mRNAs in *H. japonica* (Figure 2a). All these genes were expressed in the liver. In addition,
214 *glut2* mRNA was observed in the brain, intestine, and kidney. Expression of *g6p* mRNA was
215 detected only in the liver, whereas *pygl* was expressed in all the organs tested. *gys2* mRNA
216 was expressed in the liver and kidneys at a moderate level and in the brain and heart at a low
217 level.

218 The effects of hibernation, freezing, and thawing on the mRNA expression of *glut2*,
219 *g6p*, *pygl*, and *gys2* in the liver were analyzed by qPCR (Figure 2b–e). The *glut2* mRNA
220 expression in the hibernating, frozen, and thawed frogs was approximately 10-fold, 25-fold,
221 and 18-fold higher, respectively, than in active frogs (Figure 2b); a significant difference was
222 observed between the active and frozen groups. This suggests that, in *H. japonica*, glucose
223 transport from the liver is activated during freezing and thawing via Glut2. Rosendale et al.
224 (2014) reported that the *glut2* mRNA and protein levels in the *R. sylvatica* liver were higher in
225 the frozen group than in the hibernated group. In the thawed group, Glut2 protein level was
226 decreased to the level of hibernated group, but the mRNA level remained higher in
227 comparison of the hibernated group. At least in *H. japonica*, *glut2* mRNA expression was
228 assumed to increase before or during hibernation to some extent and to increase further when

exposed to freezing. There were no significant differences in *g6p* mRNA expression among the experimental groups (Figure 2c). As mentioned above, serum glucose levels did not differ between the active and hibernating frogs and but increased in the thawed frogs. These results suggest that the enzymic activity of G6p for glucogenesis is regulated at the translation and/or phosphorylation level, not the transcription level. In *R. sylvatica*, do Amaral et al. (2016) reported that the G6p protein level did not correlate to the mRNA expression level and presumed that distinct strategies are used to regulate the transcription and translation of this enzyme. Expression of *pygl* mRNA increased by freezing, but the fold change was much lower than that of *glut2* (Figure 2d). It is likely that Pygl protein expression is increased, and thereby glycogenolysis is activated during freezing. The increased mRNA expression of *pygl* during hibernation may facilitate a rapid response to freezing during winter. The *gys2* mRNA expression levels in the liver of hibernating and frozen groups were approximately two-fold as high as that in the active group, and it was slightly lower in thawed frogs than in hibernating and frozen frogs (Figure 2e). It is possible that increased Gys2 protein in the liver is involved in the synthesis and accumulation of glycogen in *H. japonica* during winter. In general, biochemical activities including transcriptional activity is expected to be decreased or stopped under frozen conditions. ~~However, there are reports in various species that gene expression increases by freezing. For example, do Amaral et al. (2020) found in another treefrog, *Dryophytes chrysoscels*, that 18 genes were upregulated in the liver of frozen animals compared to cold-acclimated ones.~~ However, such increase in gene expression has been reported for another treefrog, *Dryophytes chrysoscelis*, in which expression of 18 genes were upregulated in the liver of frozen animals, as compared to cold-acclimated ones (do Amara et al., 2020). Mechanisms for the upregulation of gene expression during freezing has not been explained, and further studies are necessary.

Immunohistochemistry was performed to examine the expression and localization of Glut2 in the liver. Glut2 immunoreactive signals were scarcely detected in the liver of active

frogs (Figure 3a, e) but clearly detected in the hibernating group (Figure 3b, f). HE-staining of the liver sections showed that the immunopositive signals were localized on the plasma membrane of hepatocytes in the hibernating group (Figure S2). The signal intensity increased in the frozen group (Figure 3c, g) and decreased in the thawed group (Figure 3d, h). The Glut2-antibody that had been preadsorbed with an excess amount of antigen did not immunoreact with any cells (Figure 3i, j). Specificity of the Glut2-antibody was also confirmed by western blotting. An immunopositive band was stained at ca. 55 kDa with Glut2 antibody in the liver extract of frozen *H. japonica*, and it was undetected when the antibody had been preadsorbed with the antigen peptide (Figure 3k). This histological experiment demonstrated that the abundance of Glut2 protein is increased during hibernation and further by freezing and that Glut2 protein is localized in the plasma membrane of hepatocytes. In *R. sylvatica*, immunoblot experiment showed that Glut2 protein level in the liver was increased by freezing as compared with the control hibernated group and it returned to the control level by thawing (Rosendale et al., 2014). In *H. japonica*, Glut2 immunopositive signals were fewer in the thawed group than those in the hibernated group (Figure 3b, d). It may be because of the different experimental conditions, such as temperature and time, and/or the difference of species. ~~The signal intensity was consistent with that obtained by immunoblotting using the livers of frozen or thawed *R. sylvatica* (Rosendale et al., 2014).~~

The present study indicates that glucose, along with glycerol, acts as a cryoprotectant in *H. japonica* and that its synthesis from glycogen in the liver is enhanced during hibernation and freezing. Synthesis of glucose and glycogen may be partly regulated by the mRNA expression levels of *pygl* and gys2 in the liver, respectively. Further studies are needed on the role of protein expression and enzymatic activity of these enzymes in freeze tolerance in *H. japonica*. Some freeze-tolerant amphibian species utilize urea as an additional cryoprotectant to glucose and glycerol (Storey & Storey, 2017). In *H. japonica*, our preliminary experiments showed that urea concentrations in the serum and certain tissues are not changed during

hibernation, by freezing, or by thawing as compared with the active control.

~~Endocrinological and neural mechanisms which regulate freeze tolerance in ectothermic animals has been poorly understood.~~ According to Leturque et al. (2009), insulin and glucagon, which are known as general factors to control glycogenesis and glucogenesis in the liver, affects mRNA expressions of glut2 in mammals. For freeze tolerant amphibians, there are several reports in consideration of the regulation by insulin. For example, *Rana esculenta* exhibits a marked seasonal rhythm in serum insulin, with levels falling sharply at winter's advent, whereas glucagon level is constant during the year (Schlagheck & Blüm, 1981). In *R. sylvatica*, freezing intensifies activity of β2 adrenergic receptor and decreases those of α1 and α2 adrenergic receptors in the liver (Hemmings & Storey, 1994). These facts suggest that insulin secretion regulated by autonomic nervous system may be involved in glycogen accumulation during winter and glucose release during freezing, but the details are still unclear. Hypothalamic factors, such as thyrotropin-releasing hormone and corticotropin-releasing hormone, are candidates involved in the regulation of freeze tolerance, because secretion of these hormones are enhanced by cold stimulation in mammals (~~reference~~ Zoeller, et al., 1990; Uribe, et al., 1993; Perello et al., 2007). ~~Our findings indicate that in *H. japonica,* the expression of Glut2 protein is increased during hibernation and freezing and that Glut2 mediates glucose transport from the liver during winter.~~ Further studies are needed to clarify the hormonal and/or neural regulatory mechanisms of freeze tolerance in non-mammalian animals including treefrogs.

ACKNOWLEDGMENTS

We are very grateful to Professors S. Tanaka and M. Suzuki at Shizuoka University for their valuable advice during the experiments. This work was supported in part by the Nakatsuji Foresight Foundation Research Grant to R.O.

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418 **FIGURE LEGENDS**

419 **Figure 1.** Effects of freezing and thawing on the serum glucose concentrations in *Hyla*
420 *japonica* (a) and images of periodic acid-Schiff-stained liver sections of active (b),
421 hibernating (c), frozen (d), and thawed (e) *H. japonica*.
422 (a) Data represent mean ± standard error of the mean (SEM; $n = 7$). Values with the different
423 letters are significantly different from each other (Tukey's test, $P < 0.05$).
424 (b–e) Purple-magenta color represents polysaccharides such as glycogen. Nuclei were
425 counterstained with hematoxylin. Scale bar = 100 μm.
426

427 **Figure 2.** Expressions of *glut2*, *g6p*, *pygl*, and *gys2* mRNA in various organs of active *Hyla*
428 *japonica* (a) and in the liver of active, hibernating, frozen, and thawed *H. japonica* (b–e).
429 (a) Reverse transcription-PCR products were separated on a 3% agarose gel by
430 electrophoresis and were stained with ethidium bromide. *gapdh* mRNA was used as an
431 internal control. UB, urinary bladder; FB, fat body.
432 (b–e) The mRNA levels of *glut2* (b), *g6p* (c), *pygl* (d), and *gys2* (e) in the liver were measured
433 by quantitative PCR according to the standard curve method and were normalized to *gapdh*
434 mRNA levels. Data are shown as relative values to the active group with the mean ± standard
435 error of the mean (SEM; $n = 7$). Values with the different letters are significantly different
436 from each other (Tukey's test, $P < 0.05$).
437

438 **Figure 3.** Immunohistochemistry and western blot analysis for Glut2.
439 (a–j) Liver sections from active (a), hibernating (b), frozen (c), and thawed (d) *H. japonica*
440 were stained with the anti-Glut2 antibody (red). A liver section from a frozen frog was stained
441 with the anti-Glut2 antibody preadsorbed with antigen peptide (i). Nuclei were counterstained
442 with 4′, 6-diamidino-2-phenylindole (DAPI, blue). Nomarski differential interference contrast
443 images are shown as the corresponding references: (e) to (a), (f) to (b), (g) to (c), (h) to (d),

444 and (j) to (i). Scale bar = 20 μm. (k) Plasma membrane fraction (10 μg protein) of the liver
445 from frozen *H. japonica* was electrophoresed on a 10% polyacrylamide gel, transferred to a
446 PVDF membrane, and immunoreacted with anti-Glut2 (lane 1) or antibody that had been
447 preadsorbed with the antigen peptide (lane 2). The arrow indicates *H. japonica* Glut2 protein
448 (ca. 55 kDa). Lines on the left side of the figure show molecular weight: 150, 100, 75, 50, 37,
449 20, 15, and 10 kDa, from the top.

Table 1. Primers used for cDNA cloning, RT-PCR, and qPCR

Name	Sequence (5′ to 3′)
glut2-S1	GTATTGGTCCCTCTCTGTATC
glut2-A1	TACTCCATYTCWGTAGA
glut2-5′A1	CTCATCCAAGCGTGTTCA
glut2-5′A2	CCTGCCAAACCAATAAGG
glut2-5′A3	GCGGCTGTCTGTAATTTGGG
glut2-3′S1	GCTTGGGATTGTAACTGGGATTC
glut2-3′S2	GCTGTTCTTTTGTCCAGAAAGTCC
glut2-PCRS	ATGGATTGCAGACAAACTGGGAAGG
glut2-PCRA	TACCAGCAATGACAAGGGCATGAG
g6p-S1	TYYTYTTYCCYATCTGGTTCCAYHT
g6p-A1	GCHGCACTYTTRCAGAARGA
g6p-5′A1	GATGAGGTAGAATCCCAGAGCGA
g6p -5′A2	GATTGCGTGTGATGGAATGCTT
g6p-5′A3	GACAGACAGACGCAGACCTGGAC
g6p-3′S1	GTGTCATCTCAGGCATGGTGGTGG
g6p-3′S2	ATCACACGCAATCCATCTACAAGG
g6p-PCRS	AGGTGCCTTTCCGAATGTGC
g6p-PCRA	CGTAGAAGAGCATCTCCACTTTGG
pygl-S1	GGVAAYCCHTGGGARAARGC
pygl-A1	TKGTGATBTCCCAGGCCTTG
pygl-5′A1	GGGAAATGTTCTCCGCCAGG
pygl-5′A2	GCTTCGACGTAGTCACCAAC
pygl-5′A3	CTTGCAGACCAGAGCCTCAT
pygl-3′S1	TCCCGCGTCCTGTATCCTAATG
pygl-3′S2	CCGCCTCTTTGCAGGACATCATTC
pygl-PCRS	TGTTGACCGTCTGAGGAGGATG

pygl-PCRA	TCCGACAATACACAGGTGAGCC
gys2-S1	CAYGAATTYCAGAAYYTSC
gys2-A1	GTTCCTSCATGAAACAGCCRAA
gys2-5′A1	GGGCTCGTAGTATGATGGGAAGAC
gys2-5′A2	CGTCTCGGTCCAGGATTTTGTTC
gys2-5′A3/*gys2*-PCRA	TTCTTCCCGAACTTCTCCTTCAC
gys2-3′S1/*gys2*-PCRS	CCGTCGTGGTCTTCTTCATAATG
gys2-3′S2	CGAATAACTTCAATGTGGAGACGC
gapdh-PCRS	AAAGTCATCCCAGCCCTGAACG
gapdh-PCRA	TCCAAGCGGACAGTCAAGTCAAC
Adaptor-oligo(dT) primer	GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTT
Adaptor primer	GGCCACGCGTCGACTAGTAC

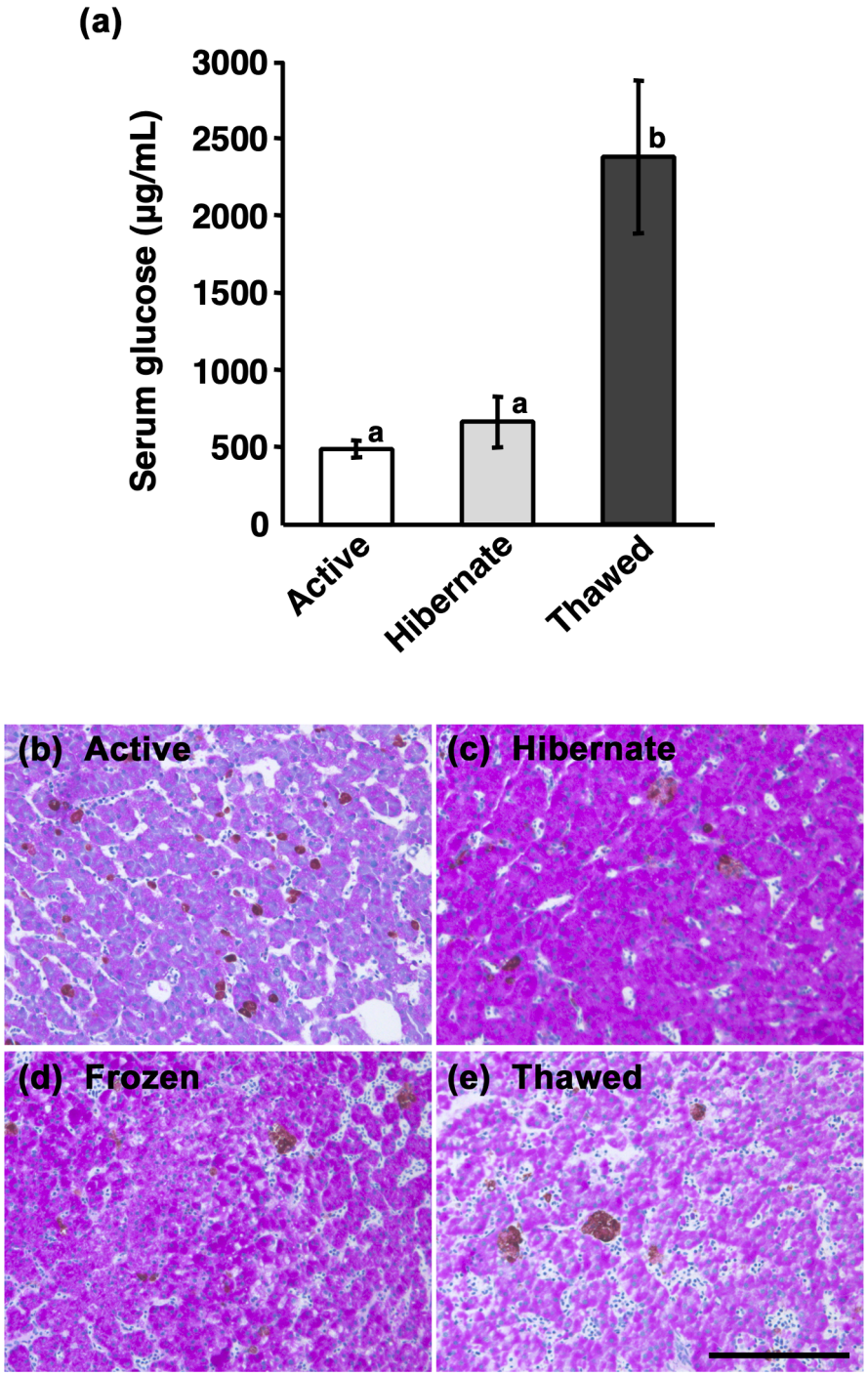

(a)
Serum glucose (µg/mL)
3000
2500
2000
1500
1000
500
0
a
a
b
Active
Hibernate
Thawed
(b) Active
(c) Hibernate
(d) Frozen
(e) Thawed

Figure 1

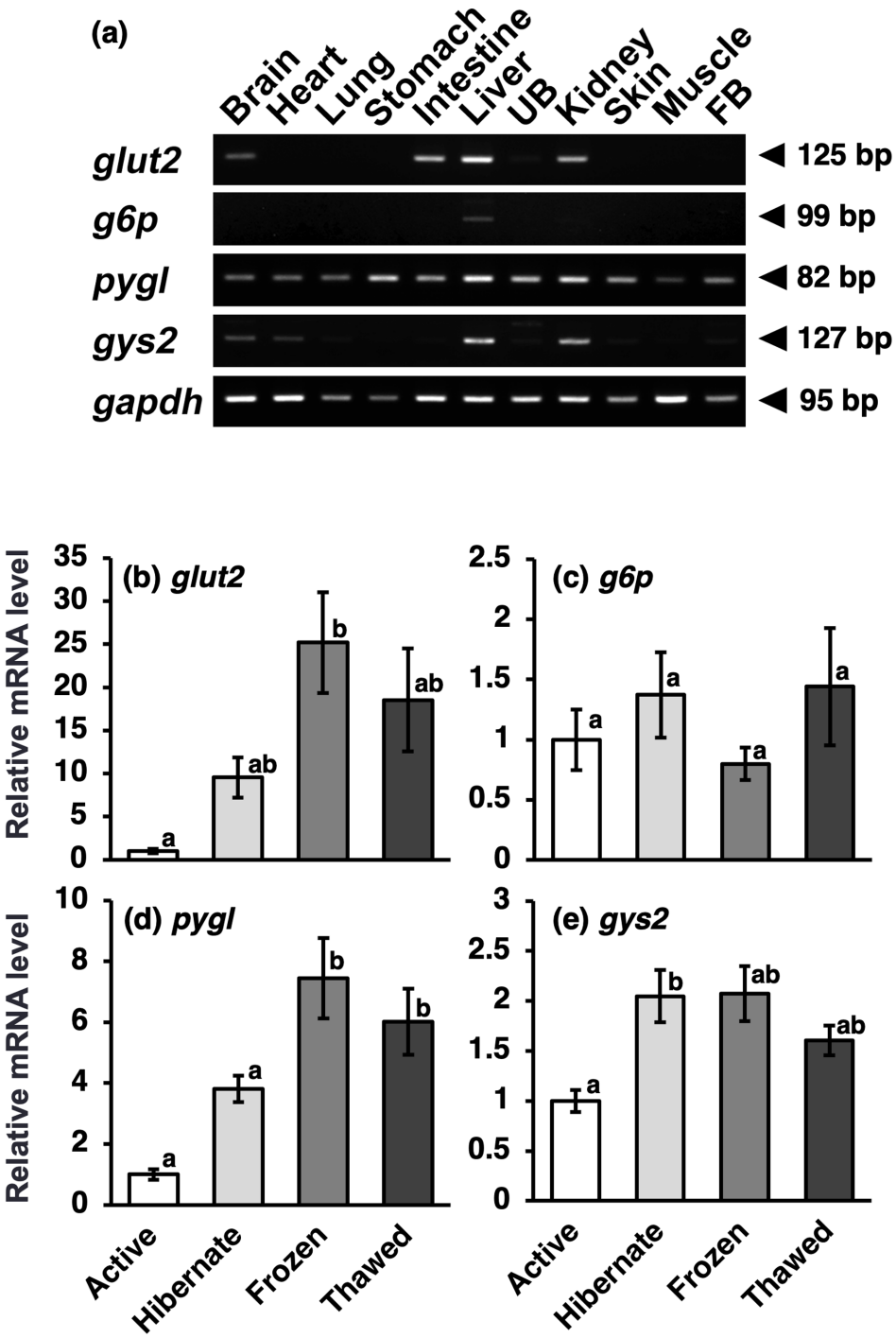
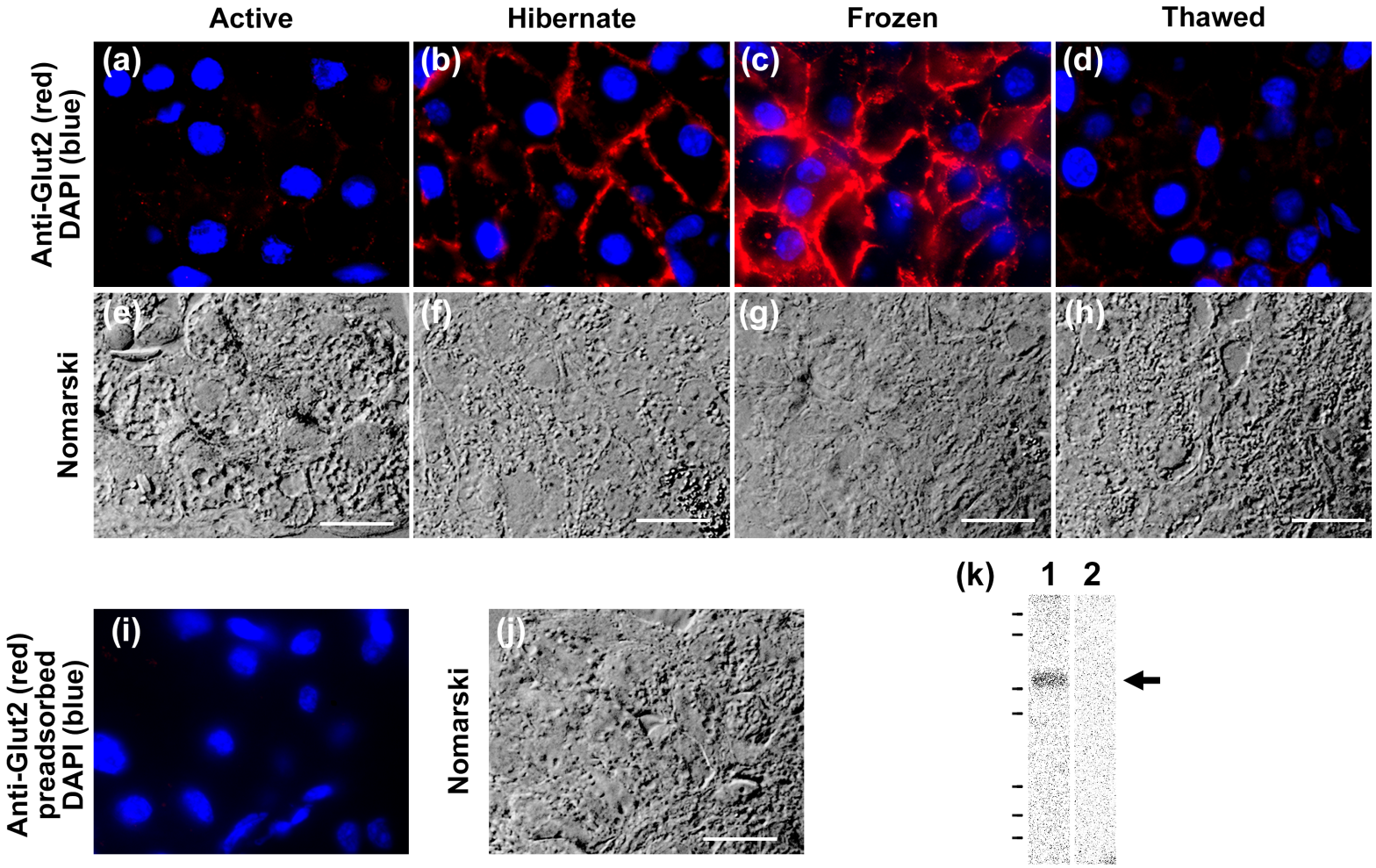

(a)
Brain
Heart
Lung
Stomach
Intestine
Liver
UB
Kidney
Skin
Muscle
FB
glut2
g6p
pygl
gys2
gapdh
125 bp
99 bp
82 bp
127 bp
95 bp
Relative mRNA level
(b) glut2
(c) g6p
(d) pygl
(e) gys2
Active
Hibernate
Frozen
Thawed

Figure 2

Figure 3


Active
Hibernate
Frozen
Thawed
Anti-Glut2 (red)
DAPI (blue)
(a)
(b)
(c)
(d)
Nomarski
(e)
(f)
(g)
(h)
Anti-Glut2 (red)
preadsorbed
DAPI (blue)
(i)
Nomarski
(j)
(k)
1
2

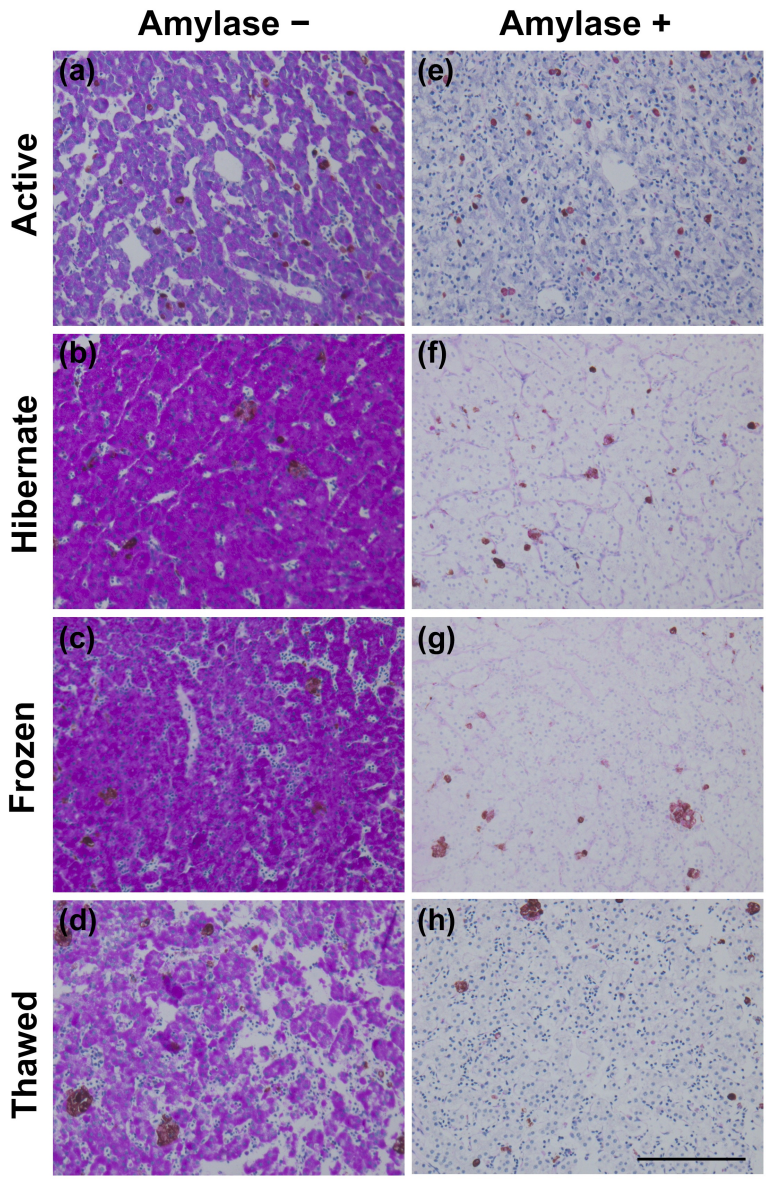


FIGURE S1
Images of periodic acid-Schiff (PAS)-stained liver sections of *Hyla japonica*. The liver sections of active (a and e), hibernating (b and f), frozen (c and g), and thawed (d and h) frogs were stained. (a–d) PAS-stained images of the liver sections. (e–h) The sections were incubated in a 1 mg/mL amylase solution before the PAS-staining. Scale bar = 50 μm.

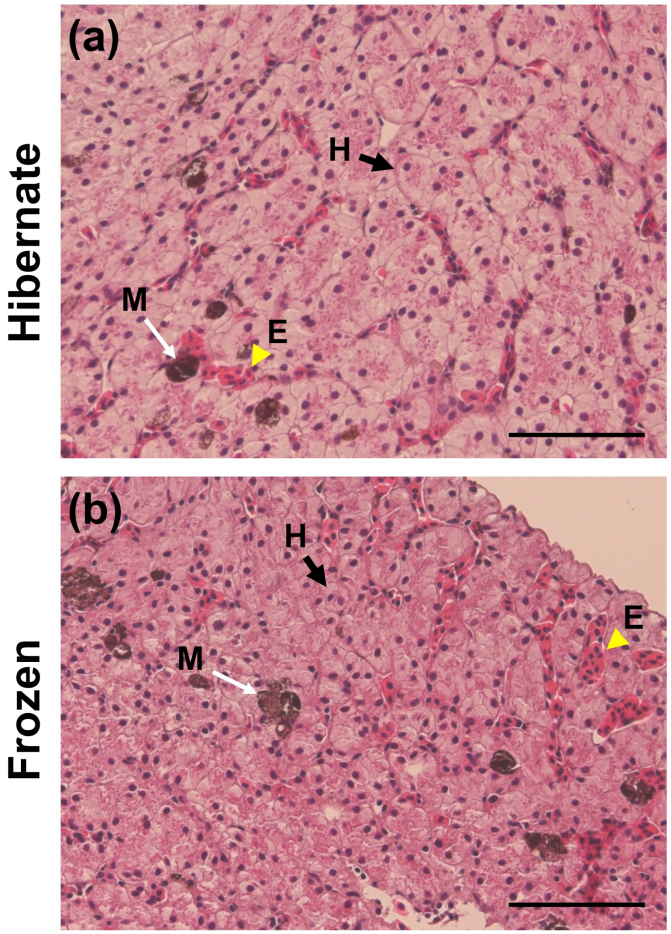


FIGURE S2
Images of hematoxylin-eosin (HE)-stained liver sections of *Hyla japonica*. The liver sections of hibernating (a) and frozen (b) frogs were stained. H (arrow in black), hepatocyte; E (arrowhead in yellow), erythrocyte, M (arrow in white), melanomacrophage aggregates. Scale bar = 100 μm.