Seasonal acclimatization and thermal acclimation induce global histone epigenetic changes in liver of bullfrog (Lithobates catesbeianus) tadpole

Seasonal acclimatization and thermal acclimation induce global histone epigenetic changes in liver of bullfrog (*Lithobates catesbeianus*) tadpole

Akinori Ishihara[a,#], Mohammad Ashrafuzzaman Sapon [b,#], Kiyoshi Yamauchi[a,b,*]

[a] *Department of Biological Science, Faculty of Science, Shizuoka University, Shizuoka 422-8529, Japan*

[b] *Department of Bioscience, Graduate School of Science and Technology, Shizuoka University, Shizuoka 422-8529, Japan*

(ishihara.akinori@shizuoka.ac.jp; sapon.btge03@gmail.com)

[#] Both of these authors contributed equally to this work.

[*]Corresponding author. Address: Department of Biological Science, Faculty of Science, Shizuoka University, Ohya 836 Suruga-ku, Shizuoka 422-8529, Japan. Fax: +81 54 238 0986. E-mail address: yamauchi.kiyoshi@shizuoka.ac.jp (K. Yamauchi)

Abbreviations: acetyl-CoA, acetyl coenzyme A; CoA-SH, free coenzyme A; ANOVA, analysis of variance; H3K9me3, trimethylated histone H3 at lysine 9; H3K36me3, trimethylated histone H3 at lysine 36; H4ac, acetylated histone H4; H3K9ac, acetylated histone H3 at lysine 9; HAT, histone acetyltransferase; HDAC, histone deacetylase; HPLC, high-performance liquid chromatography; Kac, acetylated lysine, PAGE, polyacrylamide gel electrophoresis; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; SDS, sodium dodecyl sulfate; SEM, standard error of the mean; TBS, Tris-buffered saline.

Declaration of interest: none

Abstract

The American bullfrog (*Lithobates catesbeianus*) is a eurythermal amphibian that is naturally distributed from subarctic to subtropical areas. The tadpoles of this species overwinter, in water, in cold environments. Therefore, they may have adapted to a wide range of temperatures in an active state. To understand the adaptation mechanisms to cope with low or high temperatures, we investigated global epigenetic modifications, histone variants, transcript levels of related genes, and the cellular acetyl coenzyme A (acetyl-CoA) and free CoA (CoA-SH) levels, in the livers of tadpoles collected in summer and winter and of those acclimated to 4°C and 21°C. Among epigenetic marks tested, the levels of acetylated histones and the histone variant H2A.Z were influenced by different temperature conditions. Histone acetylation levels were higher in summer than in winter and increased within 3 days of warm acclimation, whereas histone H2A.Z levels were higher in winter than in summer and decreased within 2 weeks of warm acclimation. Transcript analysis revealed that decreased expression of histone H2A.Z in warm acclimation was regulated at the transcriptional level. Acetyl-CoA levels were not correlated with those of the acetylated histones, indicating that cellular acetyl-CoA levels may not directly influence the state of histone acetylation in the tadpole liver. Such epigenetic and metabolic changes in the tadpole liver may contribute to the maintenance of energy balance during seasonal acclimatization and thermal acclimation.

Keywords: acclimation, acclimatization, acetylation, gene expression, histone, temperature, variant

1. Introduction

American bullfrogs (*Lithobates catesbeianus*) are now regarded as an invasive species, in introduced areas, and their presence represents a threat to native fauna due to their large size and aggressive behavior (Adams and Pearl, 2007; Johnson et al., 2011). Although some bullfrog tadpoles metamorphose in summer or early autumn of the hatched year, the remaining tadpoles halt the progression of metamorphosis in autumn, depending on latitude (Viparina and Just, 1975). The overwintering tadpoles become bigger in body weight and then resume metamorphosis in spring to summer of the second year (Viparina and Just, 1975). Recent studies, using toads, demonstrated that larger body size at metamorphosis enhances survival, growth and performance of metamorphosed froglets (Cabrera-Guzman et al., 2013). These observations suggest that the relatively high eurythermal properties (from subzero to ~30°C) and a complex life cycle of the bullfrogs may also have negatively impacted on native species. However, in amphibians, much less attention has been paid to the cellular responses to environmental temperature at the transcriptional level.

Several lines of evidence indicate that epigenetic processes affect gene transcription in response to extreme environmental stimuli, such as oxygen deprivation (Krivoruchko and Storey, 2010), drought (Hudson et al., 2008; Wu et al., 2013), and cold temperatures (Pinto et al., 2005; Simonet et al., 2013; Hawkins and Storey, 2018), in ectothermic vertebrates. Different epigenetic processes, or combinations thereof, are involved in these stress responses including: histone modifications (Krivoruchko and Storey, 2010; Hawkins and Storey, 2018), specific deposition of histone variants (Pinto et al., 2005; Araya et al., 2010; Simonet et al., 2013), DNA methylation (Hudson et al., 2008) and miRNA expression (Wu et al., 2013), in the liver, skeletal muscle or other tissues. Most of these studies have been performed under conditions that simulated the extreme environments where these animals may enter into estivation, hibernation or torpor, with a transition into hypometabolic states (Storey and Storey, 2004; Storey, 2015). There are, to date, few studies on epigenetic processes evaluating the effects of moderate stress conditions, such as mild temperature changes, that naturally occur in ectothermic vertebrates.

In previous studies (Mochizuki et al., 2012; Suzuki et al., 2016), in the liver, acclimated to 26°C and 4°C for short periods, we found significant differences in transcript levels for energy metabolism between the 26°C- and 4°C- acclimated bullfrog tadpoles, with changes in both mitochondrial enzyme activity and the ratio of triglycerides to cholesterol in plasma. This suggests dynamic metabolic reprogramming in response to acclimation temperature. In this study, two experimental designs were set up: (1) to elucidate the effects of seasonal acclimatization using tadpoles collected in winter and summer, and (2) to examine the effects of thermal acclimation (4°C vs. 21°C) for short and long periods (3 days or 2 weeks), on epigenetic states of the liver. The aim of this study is to assess (1) whether epigenetic mechanisms are involved in seasonal acclimatization and thermal acclimation, and (2) whether there are different epigenetic changes between the seasonal acclimatization and thermal acclimation process. We first investigated the levels of euchromatin-associated and heterochromatin-associated epigenetic marks (histone modifications and histone variants) and related gene transcripts by western blotting and reverse transcription-quantitative polymerase chain reaction (RT-qPCR), respectively. We next quantified cellular acetyl coenzyme A (acetyl-CoA) and non-esterified free CoA (CoA-SH) in the livers.

2. Materials and Methods

2.1 Reagents

3-Aminobenzoic acid ethyl ester, phenylmethylsulfonyl fluoride and CoA-SH were obtained from Sigma-Aldrich (St. Louis, MO, USA). Leupeptin, E-64, acetyl-CoA and sulfosalicylic acid were purchased from Wako (Osaka, Japan). TaqMan reverse transcription reagents kit and Power SYBR Green PCR Master Mix were from Applied Biosystems (Foster City, CA, USA). ProtoBlot AP System and secondary antibodies [alkaline phosphate conjugated anti-rabbit immunoglobulin (S3731) and anti-mouse immunoglobulin (S3721)] were obtained from Promega (Madison, WI, USA). Rabbit polyclonal antibodies against trimethylated histone H3 (synthetic

peptide within amino acids 1–100) at lysine 36 (H3K36me3; ab9050) and H2A.Z (amino acids 100–128) (ab4174) were obtained from Abcam (Tokyo, Japan), and those against trimethylated histone H3 at lysine 9 (2×-branched synthetic peptide) (H3K9me3; 07-442), acetylated histone H3 (amino acids 1–20) at lysine 9 (H3K9ac; 17-615), acetylated histone H4 (amino acids 2-19 of *Tetrahymena* histone H4) that recognizes acetylated lysines 5, 8, 12 and 16 (H4ac; 06-598), histone H3 pan (C-terminal region) (07-690), histone H4 pan (amino acids 17–28) (05-858) and histone H3.3 (a peptide specific for histone H3.3) (09-838) were from Merck Millipore (Darmstadt, Germany). Mouse monoclonal antibody against human SUMO-1 (amino acids 1–101; sc-5308) was obtained from Santa Cruz Biotechnology (Dallas, TX, USA). Mouse monoclonal antibody against acetylated lysine (a mixture of acetylated proteins) (Kac) (AAC01-S) was purchased from Cytoskeleton (Denver, CO, USA). All other reagents were of the highest grade commercially available.

2.2. Animal care and experimental design

American bullfrog tadpoles (*L. catesbeianus*), weighing 5–9 g, were collected from ponds in the southern suburbs of Shizuoka, or in Ibaraki through a commercial supplier, in Japan. The developmental stages of the tadpoles were determined according to the criteria of Taylor and Kollros (Taylor and Kollros, 1946). Tadpoles (each $n = 8$) collected in summer (in September, 2016 and July, 2017) and winter (in December, 2016 and January, 2017) were anesthetized by immersion in 0.02% 3-aminobenzoic acid ethyl ester, without acclimation to laboratory conditions. After their body weight and body length were measured, their livers were dissected. Several pieces of the liver tissues (each 20–40 mg) were snap frozen in liquid nitrogen and stored at -84°C for later use.

In warm acclimation experiments, only tadpoles collected in winter were used. They were maintained in aerated and dechlorinated tap water at 4°C, under natural lighting conditions and were fed *ad libitum* boiled spinach (given approximately 0.5 g of a frozen block/tadpole) at 9:00 AM three times per week. After acclimation to laboratory conditions at 4°C for 1 week, 32 tadpoles were divided into four groups (8

115 individuals/10 L water/group): two 4°C and two 21°C groups. For 4°C groups (control), tadpoles were
116 maintained at 4°C until Day 3 or Day 14. For 21°C groups, tadpoles were subjected to a stepped warming regime
117 of 1°C/2 h to a maximum of 6°C/day, to 21°C, over 3 days (from Day -3 to Day 0), and then maintained at 21°C
118 until Day 3 or Day 14. The mean body mass of each group was adjusted to be similar at the beginning of the
119 experiment. Half of the water volume in the aquaria (5 L) was changed 3 times per week on the next day after
120 feeding. On Day 3 or Day 14, body weight, body length and developmental stages of tadpoles were measured.
121 Tadpoles were then anesthetized with 3-aminobenzoic acid ethyl ester, the liver was collected and stored at -
122 84°C.
123 All housing and experimental procedures were conducted in accordance with the guidelines for the
124 care and use of laboratory animals of Shizuoka University (permit #29F-8) under the international guideline "Act
125 on Welfare and Management of Animals" (Ministry of Environment of Japan). Frozen rat livers (from male
126 Wistar rat strain, 28 weeks old, acclimated to 22–24°C) were provided from Dr. Sakuji Koya, Wakanyaku
127 Medical Institute Ltd. (Maebashi, Japan), with the approval from the Animal Research Committee of Wakanyaku
128 Medical Institute, Ltd. (permit Wa-2011-03).
129
130 *2.3. Preparation of liver homogenates and western blotting*
131 Frozen liver (approximately 20 mg) was homogenized in 500 μL of homogenization buffer (50 mM Tris-HCl,
132 pH 7.5, 25 mM KCl, 250 mM sucrose, 10 mM sodium butyrate, 1 mM sodium orthovanadate, 0.5 mM
133 phenylmethylsulfonyl fluoride, 2 mM leupeptin, 1.4 mM E-64) (Rumbaugh and Miller, 2011) on ice, with a
134 Polytron homogenizer. Homogenate was immediately mixed with 2× sodium dodecyl sulfate (SDS) gel-loading
135 buffer (140 mM Tris-HCl, pH 6.8, 22.4% glycerol, 6% SDS, 0.02% bromophenol blue, 10% mercaptoethanol).
136 The mixture was boiled for 5 min, and then stored at -20°C until used.
137 Western blotting was performed as previously described (Tamaoki et al., 2016). In brief, homogenates
138 containing 20–50 μg proteins were subjected to 10% or 15% SDS-polyacrylamide gel electrophoresis (PAGE).

After electrophoresis, the resolved proteins were transferred onto a polyvinylidene difluoride membrane (0.22 μm, FluoroTrans®; PALL, Port Washington, New York, USA) at 1.2 mA/cm^2 for 1 h. After blocking with 10% skim milk in Tris-buffered saline (TBS, 50 mM Tris-HCl, pH, 7.6, and 140 mM NaCl) overnight at 4°C, the membrane was then incubated for 1 h at room temperature with rabbit primary antibody directed against H3K36me3 (1:1,000), H3K9me3 (1:500), H3K9ac (1:20,000), H4ac (1:500), H2A.Z (1:1,000), H3.3 (1:1,000), H3 pan (1:25,000) or H4 pan (1:30,000), or mouse primary antibody directed against Kac (1:1,000) or SUMO-1 (1:200), in 1% skim milk/TBS. The respective antibody dilutions were first optimized in our laboratory. After incubation, membranes were rinsed three times with TBS containing 0.1% Tween 20 and then incubated with the secondary antibody (1:2,500, alkaline phosphatase-linked anti-rabbit immunoglobulin, raised in goat; or 1:7,500, alkaline phosphatase-linked anti-mouse immunoglobulin, also raised in goat) in 1% skim milk/TBS for 30 min at room temperature. Immunoblots were then developed using a detection kit containing 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium do detect alkaline phosphatase activity (ProtoBlot AP System). Band intensity was quantified using an image analyzer (LAS-4000, GE Healthcare Life Sciences, Chicago, IL, USA). To control for loading in western blots, the intensity of bands stained with antibodies directed against the modified proteins or histones in each lane were normalized against bands stained with Coomassie Brilliant Blue or bands stained with antibodies against the histones H3 pan or H4 pan.

The protein content of the homogenates was estimated by the micro-Lowry method (Jain et al., 2002) using bovine serum albumin as standard, and then read using a microplate reader.

2.4. RT-qPCR analysis

Total RNA was extracted from liver (~0.1 g) by the acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987), and its integrity was confirmed by agarose gel electrophoresis containing 2.0 M formaldehyde. RNA (200 ng) was transcribed in 10 μL of 1× Taqman RT buffer using Taqman RT reagents kit for 30 min at 48°C and then for 5 min at 95°C according to the manufacturer's instructions. Sequence data of *L.*

catesbeianus genes were obtained from public databases. Detailed information of primer sets is shown in Supplementary Table 1. Primer specificity was confirmed by BLAST searches and the appearance of a single band on gel electrophoresis. We included controls lacking cDNA templates to determine the specificity of target cDNA amplification and to assess the contamination of cDNA samples. To avoid amplification of genomic DNA, we specified forward and reverse PCR primers at neighboring exons if possible. The expression of genes of interest was estimated in triplicate using Power SYBR Green PCR Master Mix and ABI Prism 7000 sequence detection System (Applied Biosystems) with a specific primer set (each 200 nM), using the following protocol: 1 cycle of 50°C for 2 min and 95°C for 10 min, and then 40 cycles of 95°C for 15 sec and 60°C for 1 min, as previously described (Tamaoki et al., 2016). All assays gave unique dissociation curves. PCR efficiency, which was 86.3–109.3%, was determined by qPCR using RT-qPCR or RT product of total RNA as a template at different concentrations that covered 3–5 orders of magnitude. Relative quantification of transcript amounts was calculated by the comparative Cq method (Pfaffl, 2001), and the transcript amount was set to be 1.0 in the winter or 4°C-acclimated groups. We tested three candidates as reference genes: *β-actin* (*actb*), *ribosomal protein L8* (*rpl8*) and *lactate dehydrogenase B* (*ldhb*). As the Cq values for the *actb* transcript was most invariable among the experimental groups than those for the *rpl8* and *ldhb* transcripts, we used *actb* as a reference gene.

2.5. High-performance liquid chromatography (HPLC) analysis of acetyl-CoA and CoA-SH

CoA compounds were extracted according to previously described methods (Demoz et al., 1995; Shibata et al., 2012) with minor modifications. Frozen liver (approximately 50 mg) was homogenized with a Polytron homogenizer in 500 μL of ice-cold 5% sulfosalicylic acid in 50 μM dithiothreitol. The homogenate was then centrifuged at 600 × g for 10 min. The extract obtained was filtered through a disc filter (0.45 μm, 03CP045AN, Advantec, Tokyo, Japan) and then immediately used for analysis. The authentic standards for CoA-SH and acetyl-CoA were prepared in 15 mM in 5% sulfosalicylic acid containing 50 μM of dithiothreitol and stored at -84°C. The concentration was determined spectrophotometrically using ε_{260} for CoA-SH (14.6) and ε_{259} for

187 acetyl-CoA (15.4) (King et al., 1988). Individual working standards were prepared from the stock solution
188 immediately before HPLC analysis by diluting with 5% sulfosalicylic acid containing 50 μM of dithiothreitol.
189 The HPLC system consisted of a pump (model 600), a controller (model 600), a dual λ absorbance
190 detector (model 2487), and an autosampler (model 717 plus), with Empower software, from Waters (Milford,
191 MA, USA). An aliquot (20 μL) of the tissue extract was injected onto a reverse-phase C18 analytical column
192 (Mightysil RP-18 GP, 4.6 × 250 mm, 5 μm particle diameter, Kanto Chemical, Tokyo, Japan), equipped with a
193 guard column (Mightysil RP-18 GP, 4.6 × 5 mm, 5 μm) of the same packing material. The column temperature
194 was kept at 40°C. The samples were kept at 4°C inside a closed chamber of the autosampler.
195 Solvent A consisted of 100 mM sodium dihydrogen phosphate and 75 mM sodium acetate, pH 4.6.
196 Solvent B was a mixture of Solvent A and methanol (7/3, v/v). The starting mobile phase (Solvent A/Solvent
197 B = 90/10) was pumped at a flow rate of 1.0 mL/min. A linear gradient to 100% Solvent B was applied over 17
198 min and then 100% Solvent B was applied for the subsequent 20 min. This was followed by a linear gradient
199 back to the starting mobile phase over 3 min. The column was re-equilibrated with the starting mobile phase for
200 15 min before the start of the next run. Each extracted sample and the authentic standards (10 pmol) were
201 injected two times, and eluted compounds were monitored by the absorbance at 254 nm. Peaks for CoA-SH and
202 acetyl-CoA, in the liver extracts, were identified by comparison of retention times with those of authentic
203 standards determined on the same day. The retention times for CoA-SH and acetyl-CoA and their day-to-day
204 variations were 17.32 ± 0.07 (n = 5) min for CoA-SH and 20.68 ± 0.07 (n = 5) min for acetyl-CoA. The positions
205 of CoA-SH and acetyl-CoA were also confirmed using internal standards. Recovery (mean ± standard deviation)
206 of authentic standards CoA-SH and acetyl-CoA were 85.1 ± 5.2% (n = 6) and 87.8 ± 7.4% (n = 6) when they
207 were added to the liver extracts. CoA compounds in the liver extracts were quantified using the linear calibration
208 curves [$y = 0.5205x - 0.0217$, $r^2 = 0.99993$, for CoA-SH ranging from 5 to 60 pmol; and $y = 0.5334x - 0.321$,
209 $r^2 = 0.99995$, for acetyl-CoA ranging from 3.4 to 43 pmol. y, peak area ($\mu Vsec \times 10^{-3}$); x, amount of CoA
210 compounds (pmol), r^2, coefficient of determination] of the authentic standards. The lower limits of detection for

CoA-SH and acetyl-CoA were 5 and 3.4 pmol, respectively.

2.6. Statistical analysis

All assay data are presented as mean ± standard error of the mean (SEM). Differences between two groups were analyzed by the Student's *t*-test. Differences between groups were analyzed by a one-way or a two-way analysis of variance (ANOVA), with Scheffe's or Fisher's test for multiple comparisons. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Morphological parameters of seasonally acclimatized and thermally acclimated tadpoles

There were no significant differences in body length, body weight and developmental stages between the winter and summer tadpoles, nor between the 4°C- and 21°C-acclimated tadpoles. Liver weight of the summer tadpoles was significantly lower than that of the winter tadpoles ($p < 0.05$) although no significant difference in liver weight was detected between the 4°C- and 21°C-acclimated tadpoles. The hepatosomatic index was 0.02–0.03, with no significant difference between the summer and winter tadpoles nor between the 4°C- and 21°C-acclimated tadpoles (data not shown).

3.2. Sumoylated and acetylated proteins in liver homogenates of seasonally acclimatized tadpoles

The intensities of three bands (approximately 110 kDa, 33 kDa and 24 kDa), immunoreactive to anti-SUMO-1, were higher in the liver homogenates of the winter tadpoles than in those of the summer tadpoles, whereas the intensity of sumoylated 29 kDa protein was higher in the summer homogenates than in the winter homogenates (Fig. 1). In the case of acetylated lysine on proteins, acetylated levels of 18-kDa and 15-kDa proteins were higher in the summer homogenates than in the winter homogenates. There were

no significant differences observed in CBB staining pattern between the winter and summer homogenates.

3.3. Acetylated and methylated histones in liver homogenates of seasonally acclimatized and thermally acclimated tadpoles

Amounts of H3K9ac histones were significantly higher in the summer homogenates than in the winter homogenates, however, no significant differences were detected in the amounts of the other histone modifications tested (H3K9me3, H3K36me3 and H4ac) between the winter and summer homogenates (Fig. 2A). In the liver homogenates of tadpoles that were experimentally acclimated to 21°C for 3 days, the amounts of H3K9ac and H4ac were significantly increased, compared with those of the 4°C-acclimated tadpoles (Fig. 2B). After 2 weeks of acclimation, increased levels of H3K9ac was maintained in the 21°C-acclimated tadpoles (Fig. 2B).

3.4. Transcript levels of histone acetyltransferases (HATs) and deacetylases (HDACs) in livers of seasonally acclimatized and thermally acclimated tadpoles

In the liver of acclimatized tadpoles (Fig. 3A), all of the transcripts tested, except for *ncoa1* and *p300*, were significantly less abundant in summer than in winter. On Day 3 of thermal acclimation (Fig. 3B), the transcript levels of one (*p300*) out of six HAT genes were significantly lower in the 21°C- than in the 4°C-acclimated tadpoles, whereas there was no significant difference in transcript level of three HDAC genes between the 21°C- and 4°C-acclimated tadpoles. After 2 weeks of acclimation (Fig. 3B), transcript amounts of five HAT genes (*crebbp*, *kat2a*, *ncoa2*, *ncoa3* and *p300*) and two HDAC genes (*hdac1* and *sirt1*) were significantly lower in the 21°C- than in the 4°C-acclimated tadpoles. As positive controls for thermal response at the transcription level, levels of three genes that are known to be induced by cold stress (*cirp* and *scd1*) (Saito et al., 2000; Gracey et al., 2004; Mochizuki et al., 2012; Suzuki et al., 2016) and both cold and heat stresses (hsp90) (Podrabsky and Somero, 2004; Buckley et al., 2006; Teigen et al., 2015) in

ectothermic vertebrates, were also investigated. All of these transcripts were significantly lower in the summer-acclimatized tadpoles than in the winter-acclimatized tadpoles. In thermally-acclimated tadpoles, only the *cirp* transcript and the *cirp* and *hsp90* transcripts were significantly down-regulated in the 21°C-acclimated tadpoles than in the 4°C-acclimated tadpoles.

3.5. Histone variants in liver homogenates of seasonally acclimatized and thermally acclimated tadpoles

The level of histone H2A.Z in the liver homogenates was significantly lower in the summer tadpoles than in the winter tadpoles (Fig. 4A). A similar result was obtained in the 2-week acclimation to 21°C compared with that to 4°C (Fig. 4B), but not in 3-day acclimation (Fig. 4B). No significant differences were detected in amounts of the histone variant H3.3 between the winter and summer tadpoles and between the 4°C- and 21°C-acclimated tadpoles.

3.6. Transcript levels of histone variants in livers of seasonally acclimatized and thermally acclimated tadpoles

In the seasonal acclimatization study (Fig. 5A), the amounts of *h2afz* (for histone H2A.Z1) and *h2afy* (for histone macroH2A.1) transcripts were significantly lower in the summer tadpoles than in the winter tadpoles. In the thermal acclimation study (Figs. 5B), the amount of *h2afz* transcript was significantly lower in the 21°C-acclimated than in the 4°C-acclimated tadpoles, only at 2 weeks of acclimation.

3.7. Cellular acetyl-CoA and CoA-SH content in livers of seasonally acclimatized and thermally acclimated tadpoles

Hepatic acetyl-CoA content of the tadpoles was variable (4.01–13.29 nmol/g wet weight), depending on habitat or rearing temperatures and acclimation periods. These values were 23–77% of those of the rat liver samples (17.30 nmol/g wet weight). The CoA-SH content of the tadpole livers (4.64–9.77 nmol/g wet

283 weight) was only 3–6% of those of the rat livers (151. 60 nmol/g wet weight) (Table 2). The acetyl-CoA
284 and CoA-SH content of the rat liver obtained in this study were comparable to those published in previous
285 reports (Williamson and Brosnan, 1974; Demoz et al., 1995; Shibata et al., 2012; Shurubor et al., 2017).
286 Acetyl-CoA content of the summer tadpoles was approximately two-thirds of that of the winter
287 tadpoles, although there was no significant difference in CoA-SH content between the winter and summer
288 tadpoles, with a parallel change in the ratio of acetyl-CoA to CoA-SH (1.53 in winter vs 0.89 in summer).
289 In acclimation for 3 days, the acetyl-CoA and CoA-SH content of the 21°C-acclimated tadpoles
290 decreased significantly to 71% and 73%, respectively, of those of the 4°C-acclimated tadpoles, while
291 maintaining a constant ratio of acetyl-CoA to CoA-SH (2.1–2.2 nmol/g wet weight). However, in
292 acclimation for 2 weeks, we could not detect any significant changes in the acetyl-CoA (4.9–5.4 nmol/g wet
293 weight) and CoA-SH (4.0–4.6 nmol/g wet weight) content nor in the ratio of acetyl-CoA to CoA-SH (0.8–
294 0.9) between the 4°C- and 21°C-acclimated tadpoles.
295
296
297 **4. Discussion**
298 In this study, we demonstrate that seasonal acclimatization and thermal acclimation affect global epigenetic
299 states of histones in the liver of bullfrog tadpoles, with a complex pattern of acetyl-CoA and CoA-SH
300 content. The quantities of acetylated histones, typical euchromatin-associated epigenetic marks (Strahl and
301 Allis, 2000; Jenuwein and Allis, 2001), were higher, and the amount of histone H2A.Z, which is involved
302 in various cellular processes including euchromatic activation, hetrerochromatic silencing or transcriptional
303 memory (Subramanian et al., 2015), was lower, in the tadpoles that were acclimatized or acclimated to
304 warm temperatures. Acetylated histone levels changed quickly (within 3 days), while histone H2A.Z levels
305 changed slowly (within 2 weeks), during warm acclimation. Transcript analysis revealed that the changes in
306 histone H2A.Z levels could be explained by transcriptional control while the changes in acetylated histone

levels could not be explained by changes at transcriptional level. At least two different epigenetic processes may therefore be involved in seasonal acclimatization and thermal acclimation.

4.1. Different amounts of epigenetic marks detected in livers of seasonally acclimatized and thermally acclimated tadpoles

Cellular proteins may be restrictedly sumoylated or acetylated in the livers of seasonally acclimatized tadpoles. Some differences were detected in the amount of these modified proteins in the livers when comparing the winter and summer tadpoles. In summer tadpoles, we detected a rise in acetylation levels of only the 15- and 18-kDa proteins, corresponding to core histones (von Holt et al., 1989). In contrast, mammalian cells or tissues exposed to heat stress (Saitoh and Hinchey, 2000) or being in hibernation (Lee et al., 2007) have many sumoylated proteins that were clearly detected as a ladder of multiple bands of 100- to 200-kDa on western blotting. In *Xenopus laevis* larvae, acetylation of 16- to 18-kDa core-histones, concomitant with 230- and 100-kDa proteins, accumulated during post-embryonic development (Tsuchiya et al., 2014). In ground squirrels, many mitochondrial proteins were acetylated during hibernation, suggesting mitochondria-based metabolic reprogramming (Hindle et al., 2014). However, we could not detect such heavily sumoylated or acetylated high-molecular-weight-proteins in tadpole livers. Sumoylation is induced by several environmental stressors including heat, osmotic, hypoxic, oxidative and genotoxic shocks (Saitoh and Hinchey, 2000; Tempe et al., 2008), and controls the intracellular traffic between the cytoplasm and nucleus, cell signaling, DNA repair, cell cycle and transcription (Niskanen and Palvimo, 2017). Acetylation of extranuclear (mainly mitochondrial) proteins is principally associated with their functional inactivation in mammalian cells (Drazic et al., 2016). Results from our study and these previously published observations suggest that sumoylation and acetylation of extranuclear proteins may contribute less to the seasonal acclimatization processes in tadpoles.

Core-histones may be major targets for protein acetylation in the liver of seasonally acclimatized

and thermally acclimated tadpoles. Enhanced histone acetylation (H3K9ac and/or H4ac) may facilitate transcriptional activation through changes in chromatin structure from a compact to a more relaxed state (Strahl and Allis, 2000; Jenuwein and Allis, 2001) in the liver of summer-acclimatized and the 21°C-acclimated tadpoles, who had relatively higher metabolic and locomotion activities. Meanwhile, a heterochromatin-associated epigenetic mark for transcriptional suppression, H3K9me3, may play a less important role in seasonal acclimatization or in thermal acclimation, since no global changes in H3K9me3 level between the acclimatized, nor between the acclimated groups were observed.

Higher amounts of histone H2A.Z detected in winter acclimatization and in 4°C acclimation for 2 weeks suggest that histone H2A.Z may be involved in the regulation of transcriptional activity in cold environments. Previous reports suggested a major role of histone H2A.Z in transcriptional responses to fluctuations of environmental temperature or seasonal variations in both a plant species (*Arabidopsis thaliana*) (Kumar and Wigge, 2010) and a fish species (*Cyprinus carpio*) (Simonet et al., 2013). Therefore, the role of histone H2A.Z as a thermoregulator or thermosensor (Talbert and Henikoff, 2014) may be a fundamental function that is common to higher plants and ectothermic vertebrates. Interestingly, in the short-term acclimation study (for 3 days), we could not detect significant changes in the amount of histone H2A.Z between the 4°C- and 21°C- tadpoles. Adaptive regulation mediated by changes in histone H2A.Z amount may require a prolonged period, longer than 3 days, which is different from the rapid response of histone acetylation, occurring within 3 days.

As we detected global, and therefore average, epigenetic changes at the cellular level using western blot analyses, more dramatic fluctuations in acetylated histone and histone H2A.Z levels might occur in specific gene regions. Therefore, more sensitive analyses such as chromatin immunoprecipitation assays, will be necessary to identify the quantitative and qualitative changes in histone modifications or replacement of histone variants on specific genes in the liver of the summer-acclimatized and 21°C-acclimated tadpoles.

355

356 *4.2. Transcript analysis of genes responsible for epigenetic changes*

357 Among the genes we analyzed, only the HDAC (*hdac1* and *sirt1*) and histone H2.Z (*h2afz*) genes were
358 possible candidates responsible for epigenetic changes. To clarify the involvement of HDACs in the
359 changes in the amount of acetylated histones, we investigated the effect of HDAC inhibitors (trichostatin A
360 and nicotinamide) on the amount of H3K9ac in the livers of the tadpoles acclimated to 4°C and 21°C for 3
361 days. Trichostatin A (100 nM) had effects on histone acetylation in neither the 4°C- nor 21°C-acclimated
362 tadpoles. However, nitotinamide (2 mM) significantly enhanced the amount of H3K9ac only in the 21°C-
363 acclimated tadpoles (data not shown), suggesting that NAD^+-dependent HDACs, sirtuins, were active in the
364 liver, at least of the 21°C-acclimated tadpoles. From our observations, we conclude that the temperature-
365 dependent changes in the amounts of acetylated histones may not be due to fluctuations in transcript levels
366 of HAT and/or HDAC genes. Plausible alternatives that should be tested in future are: (1) temperature-
367 dependency of HAT activities, (2) various metabolites that influence the HAT or HDAC activities, e.g.,
368 concentrations of acetyl-CoA, NAD^+, other metabolites, or their derivatives (Yang and Sauve, 2006;
369 Vogelauer et al., 2012; Shimazu et al., 2013; Lee et al., 2014), and (3) post-translational modifications of
370 these enzymes or enzyme-associated factors (Santos-Rosa et al., 2003; Marino et al., 2014; Carrer et al.,
371 2017).

372 Analysis of transcript amounts for histone variants revealed that seasonal or temperature-
373 dependent changes in the level of histone H2A.Z are, at least in part, transcriptionally controlled, in
374 agreement with a previous report in seasonally acclimatized carp (Simonet et al., 2013). A rapid response of
375 acetylated histone levels and a relatively slow response of histone H2A.Z levels to temperature changes
376 may reflect the different mechanisms underlying their control. Although the transcript amount of *h2afy* (for
377 histone macroH2A.1) was lower in the livers of the summer tadpoles than in those of the winter tadpoles,
378 we could not detect a specific immunolabelled signal corresponding to histone macroH2A. In *C. carpio*,

379 histone macroH2A expression is increased in winter, accompanied by the enrichment of condensed
380 chromatin and hypermethylation of DNA, suggesting that it may play a role in gene repression in winter
381 acclimatization (Pinto et al., 2005). A recent report indicated that two types of macroH2A (H2A.1 and
382 H2A.2) antagonistically participate in the transcriptional regulation of the ribosomal cistron during seasonal
383 acclimatization (Araya et al., 2010). Therefore, it remains to be elucidated whether histone macroH2A is
384 involved in transcriptional regulation in bullfrog tadpoles, at least during seasonal acclimatization.
385
386 *4.3. Cellular acetyl-CoA content in liver of seasonally acclimatized and thermal acclimated tadpoles*
387 Cellular acetyl-CoA and CoA-SH content was altered in seasonal acclimatization and thermal acclimation
388 in a complex manner. Seasonal acclimatization primarily changed the acetyl-CoA content (or the ratio of
389 acetyl-CoA to CoA-SH), which was higher in winter than in summer. After 3 days of acclimation, both
390 cellular acetyl-CoA and CoA-SH contents were higher at 4ºC than at 21°C. The pooled quantity of summed
391 acetyl-CoA and CoA-SH decreased with increasing period of acclimation, regardless of the acclimation
392 temperature. The ratio of acetyl-CoA to CoA-SH was the same between the 4°C- and 21°C-acclimated
393 tadpoles, but different between Day 3 time-point (approximately 2.1) and Day 14 time-point (0.8–0.9). It is
394 generally accepted that protein acetylation levels, including histone acetylation levels, change in parallel
395 with acetyl-CoA levels depending on nutritional status (Pietrocola et al., 2015). For example, starvation
396 induces deprivation of acetyl-CoA and protein or histone deacetylation in cellular homogenates and
397 cytosolic fractions of cultured mammalian cells (Lee et al., 2014; Marino et al., 2014). Reducing the
398 temperature from 25ºC to 18°C decreased histone acetylation through the suppression of ATP-citrate lyase
399 activity in fruit flies (Peleg et al., 2016). However, histone acetylation levels were negatively correlated
400 with the acetyl-CoA content in the seasonally acclimatized and 3-day thermally acclimated tadpoles, with
401 no correlation in 2-week thermally acclimated tadpoles, suggesting several layers of mechanisms by which
402 energy metabolites are modified in response to seasonal acclimatization and thermal acclimation.

The cellular acetyl-CoA content in the tadpole liver varied from 4 to 13.3 nmol/g wet weight, which represents 23% to 77% of that seen in rat liver, whereas the cellular CoA-SH content in the tadpole liver was only 3–6% of that found in rat liver. The relatively small quantities of acetylated proteins (mainly histones) in the tadpole liver homogenates, compared with those in the rat liver homogenates, may be due to the small size of the acetyl-CoA and CoA-SH pool. Currently, there is a lack of information regarding the regulation of cellular acetyl-CoA and CoA-SH content in ectothermic vertebrates in response to environmental stresses.

In conclusion, we report here two different types of epigenetic changes in response to seasonal acclimatization and thermal acclimation in the liver of bullfrog tadpoles. One is histone acetylation, which was higher in the summer or 21°C-acclimated tadpoles. Secondly, histone variant H2A.Z levels changed, being higher in the winter or 4°C-acclimated tadpoles. Histone acetylation levels changed within 3 days of acclimation, not through transcriptional control, whereas histone H2A.Z levels changed within 2 weeks of acclimation through transcriptional activation. Cellular acetyl-CoA content or the ratio of acetyl-CoA to CoA-SH was not correlated with the amounts of acetylated histones, suggesting that histone acetylation may be controlled by factors other than cellular acetyl-CoA or the ratio of acetyl-CoA to CoA-SH.

Acknowledgments

We are grateful to Dr. M. Okada, Ms. A. Mori, and Mr. K. Nakahara for the technical assistance with western blotting and real-time RT-qPCR.

References

Adams, M.J., Pearl, C.A., 2007. Problems and opportunities managing invasive bullfrogs: is there any hope? In: (Gherardi, F. Ed.) Biological invaders in inland waters: Profiles, distribution, and threats. vol. 2. Invading Nature, Springer, Dordrecht, pp. 679-693.

Araya, I., Nardocci, G., Morales, J., Vera, M., Molina, A., Alvarez, M., 2010. MacroH2A subtypes contribute antagonistically to the transcriptional regulation of the ribosomal cistron during seasonal acclimatization of the carp fish. Epigenetics Chromatin 3, 14.

Buckley, B.A., Gracey, A.Y., Somero, G.N., 2006. The cellular response to heat stress in the goby Gillichthys mirabilis: a cDNA microarray and protein-level analysis. J. Exp. Biol. 209, 2660-2677.

Cabrera-Guzman, E., Crossland, M.R., Brown, G.P., Shine, R., 2013. Larger body size at metamorphosis enhances survival, growth and performance of young cane toads (*Rhinella marina*). PLoS One 8, e70121.

Carrer, A., Parris, J.L., Trefely, S., Henry, R.A., Montgomery, D.C., Torres, A., Viola, J.M., Kuo, Y.M., Blair, I.A., Meier, J.L., Andrews, A.J., Snyder, N.W., Wellen, K.E., 2017. Impact of a high-fat diet on tissue acyl-CoA and histone acetylation levels. J. Biol. Chem. 292, 3312-3322.

Chomczynski, P., Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162, 156-159.

Demoz, A., Garras, A., Asiedu, D.K., Netteland, B., Berge, R.K., 1995. Rapid method for the separation and detection of tissue short-chain coenzyme A esters by reversed-phase high-performance liquid chromatography. J. Chromatogr. B Biomed. Appl. 667, 148-152.

Drazic, A., Myklebust, L.M., Ree, R., Arnesen, T., 2016. The world of protein acetylation. Biochim. Biophys. Acta 1864, 1372-1401.

Gracey, A.Y., Fraser, E.J., Li, W., Fang, Y., Taylor, R.R., Rogers, J., Brass, A., Cossins, A.R., 2004. Coping with cold: An integrative, multitissue analysis of the transcriptome of a poikilothermic vertebrate.

Proc. Natl. Acad. Sci. U. S. A. 101, 16970-16975.

Hawkins, L.J., Storey, K.B., 2018. Histone methylation in the freeze-tolerant wood frog (*Rana sylvatica*). J. Comp. Physiol. B 188, 113-125.

Hindle, A.G., Grabek, K.R., Epperson, L.E., Karimpour-Fard, A., Martin, S.L., 2014. Metabolic changes associated with the long winter fast dominate the liver proteome in 13-lined ground squirrels. Physiol. Genomics 46, 348-361.

Hudson, N.J., Lonhienne, T.G., Franklin, C.E., Harper, G.S., Lehnert, S.A., 2008. Epigenetic silencers are enriched in dormant desert frog muscle. J. Comp. Physiol. B 178, 729-734.

Jain, S., Sharma, S., Gupta, M.N., 2002. A microassay for protein determination using microwaves. Anal. Biochem. 311, 84-86.

Jenuwein, T., Allis, C.D., 2001. Translating the histone code. Science 293, 1074-1080.

Johnson, P.T., McKenzie, V.J., Peterson, A.C., Kerby, J.L., Brown, J., Blaustein, A.R., Jackson, T., 2011. Regional decline of an iconic amphibian associated with elevation, land-use change, and invasive species. Conserv. Biol. 25, 556-566.

King, M.T., Reiss, P.D., Cornell, N.W., 1988. Determination of short-chain coenzyme A compounds by reversed-phase high-performance liquid chromatography. Methods Enzymol. 166, 70-79.

Krivoruchko, A., Storey, K.B., 2010. Epigenetics in anoxia tolerance: a role for histone deacetylases. Mol. Cell. Biochem. 342, 151-161.

Kumar, S.V., Wigge, P.A., 2010. H2A.Z-containing nucleosomes mediate the thermosensory response in *Arabidopsis*. Cell 140, 136-147.

Lee, J.V., Carrer, A., Shah, S., Snyder, N.W., Wei, S., Venneti, S., Worth, A.J., Yuan, Z.F., Lim, H.W., Liu, S., Jackson, E., Aiello, N.M., Haas, N.B., Rebbeck, T.R., Judkins, A., Won, K.J., Chodosh, L.A., Garcia, B.A., Stanger, B.Z., Feldman, M.D., Blair, I.A., Wellen, K.E., 2014. Akt-dependent metabolic reprogramming regulates tumor cell histone acetylation. Cell Metab. 20, 306-319.

475 Lee, Y.J., Miyake, S., Wakita, H., McMullen, D.C., Azuma, Y., Auh, S., Hallenbeck, J.M., 2007. Protein
476 SUMOylation is massively increased in hibernation torpor and is critical for the cytoprotection
477 provided by ischemic preconditioning and hypothermia in SHSY5Y cells. J. Cereb. Blood Flow
478 Metab. 27, 950-962.
479 Marino, G., Pietrocola, F., Eisenberg, T., Kong, Y., Malik, S.A., Andryushkova, A., Schroeder, S., Pendl, T.,
480 Harger, A., Niso-Santano, M., Zamzami, N., Scoazec, M., Durand, S., Enot, D.P., Fernandez, A.F.,
481 Martins, I., Kepp, O., Senovilla, L., Bauvy, C., Morselli, E., Vacchelli, E., Bennetzen, M., Magnes,
482 C., Sinner, F., Pieber, T., Lopez-Otin, C., Maiuri, M.C., Codogno, P., Andersen, J.S., Hill, J.A.,
483 Madeo, F., Kroemer, G., 2014. Regulation of autophagy by cytosolic acetyl-coenzyme A. Mol.
484 Cell 53, 710-725.
485 Mochizuki, K., Goda, T., Yamauchi, K., 2012. Gene expression profile in the liver of *Rana catesbeiana*
486 tadpoles exposed to low temperature in the presence of thyroid hormone. Biochem. Biophys. Res.
487 Commun. 420, 845-850.
488 Niskanen, E.A., Palvimo, J.J., 2017. Chromatin SUMOylation in heat stress: To protect, pause and
489 organise?: SUMO stress response on chromatin. Bioessays 39, 1600263.
490 Peleg, S., Feller, C., Forne, I., Schiller, E., Sevin, D.C., Schauer, T., Regnard, C., Straub, T., Prestel, M.,
491 Klima, C., Schmitt Nogueira, M., Becker, L., Klopstock, T., Sauer, U., Becker, P.B., Imhof, A.,
492 Ladurner, A.G., 2016. Life span extension by targeting a link between metabolism and histone
493 acetylation in *Drosophila*. EMBO Rep. 17, 455-469.
494 Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic
495 Acids Res. 29, e45.
496 Pietrocola, F., Galluzzi, L., Bravo-San Pedro, J.M., Madeo, F., Kroemer, G., 2015. Acetyl coenzyme A: a
497 central metabolite and second messenger. Cell Metab. 21, 805-821.
498 Pinto, R., Ivaldi, C., Reyes, M., Doyen, C., Mietton, F., Mongelard, F., Alvarez, M., Molina, A., Dimitrov,

S., Krauskopf, M., Vera, M.I., Bouvet, P., 2005. Seasonal environmental changes regulate the expression of the histone variant macroH2A in an eurythermal fish. FEBS Lett. 579, 5553-5558.

Podrabsky, J.E., Somero, G.N., 2004. Changes in gene expression associated with acclimation to constant temperatures and fluctuating daily temperatures in an annual killifish *Austrofundulus limnaeus*. J. Exp. Biol. 207, 2237-2254.

Rumbaugh, G., Miller, C.A., 2011. Epigenetic changes in the brain: measuring global histone modifications. Methods Mol. Biol. 670, 263-274.

Saito, T., Sugimoto, K., Adachi, Y., Wu, Q., Mori, K.J., 2000. Cloning and characterization of amphibian cold inducible RNA-binding protein. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 125, 237-245.

Saitoh, H., Hinchey, J., 2000. Functional heterogeneity of small ubiquitin-related protein modifiers SUMO-1 versus SUMO-2/3. J. Biol. Chem. 275, 6252-6258.

Santos-Rosa, H., Valls, E., Kouzarides, T., Martinez-Balbas, M., 2003. Mechanisms of P/CAF auto-acetylation. Nucleic Acids Res. 31, 4285-4292.

Shibata, K., Nakai, T., Fukuwatari, T., 2012. Simultaneous high-performance liquid chromatography determination of coenzyme A, dephospho-coenzyme A, and acetyl-coenzyme A in normal and pantothenic acid-deficient rats. Anal. Biochem. 430, 151-155.

Shimazu, T., Hirschey, M.D., Newman, J., He, W., Shirakawa, K., Le Moan, N., Grueter, C.A., Lim, H., Saunders, L.R., Stevens, R.D., Newgard, C.B., Farese, R.V. Jr., de Cabo, R., Ulrich, S., Akassoglou, K., Verdin, E., 2013. Suppression of oxidative stress by β-hydroxybutyrate, an endogenous histone deacetylase inhibitor. Science 339, 211-214.

Shurubor, Y.I., D'Aurelio, M., Clark-Matott, J., Isakova, E.P., Deryabina, Y.I., Beal, M.F., Cooper, A.J.L., Krasnikov, B.F., 2017. Determination of coenzyme A and acetyl-coenzyme A in biological samples using HPLC with UV detection. Molecules 22, 1388.

Simonet, N.G., Reyes, M., Nardocci, G., Molina, A., Alvarez, M., 2013. Epigenetic regulation of the ribosomal cistron seasonally modulates enrichment of H2A.Z and H2A.Zub in response to different environmental inputs in carp (*Cyprinus carpio*). Epigenetics Chromatin 6, 22.

Storey, K.B., 2015. Regulation of hypometabolism: insights into epigenetic controls. J. Exp. Biol. 218, 150-159.

Storey, K.B., Storey, J.M., 2004. Metabolic rate depression in animals: transcriptional and translational controls. Biol. Rev. Camb. Philos. Soc. 79, 207-233.

Strahl, B.D., Allis, C.D., 2000. The language of covalent histone modifications. Nature 403, 41-45.

Subramanian, V., Fields, P.A., Boyer, L.A., 2015. H2A.Z: a molecular rheostat for transcriptional control. F1000Prime Rep. 7, 01.

Suzuki, S., Awai, K., Ishihara, A., Yamauchi, K., 2016. Cold temperature blocks thyroid hormone-induced changes in lipid and energy metabolism in the liver of *Lithobates catesbeianus* tadpoles. Cell Biosci. 6, 19.

Talbert, P.B., Henikoff, S., 2014. Environmental responses mediated by histone variants. Trends Cell Biol. 24, 642-650.

Tamaoki, K., Okada, R., Ishihara, A., Shiojiri, N., Mochizuki, K., Goda, T., Yamauchi, K., 2016. Morphological, biochemical, transcriptional and epigenetic responses to fasting and refeeding in intestine of *Xenopus laevis*. Cell Biosci. 6, 2.

Taylor, A.C., Kollros, J.J., 1946. Stages in the normal development of *Rana pipiens* larvae. Anat. Rec. 94, 7-13.

Teigen, L.E., Orczewska, J.I., McLaughlin, J., O'Brien, K.M., 2015. Cold acclimation increases levels of some heat shock protein and sirtuin isoforms in threespine stickleback. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 188, 139-147.

Tempe, D., Piechaczyk, M., Bossis, G., 2008. SUMO under stress. Biochem. Soc. Trans. 36, 874-878.

547 Tsuchiya, Y., Pham, U., Hu, W., Ohnuma, S., Gout, I., 2014. Changes in acetyl CoA levels during the early
548 embryonic development of *Xenopus laevis*. PLoS One 9, e97693.
549 Viparina, S., Just, J.J., 1975. The life period, growth and differentiation of *Rana catesbeiana* larvae
550 occurring in nature. Copeia 1975, 103-109.
551 Vogelauer, M., Krall, A.S., McBrian, M.A., Li, J.Y., Kurdistani, S.K., 2012. Stimulation of histone
552 deacetylase activity by metabolites of intermediary metabolism. J. Biol. Chem. 287, 32006-32016.
553 von Holt, C., Brandt, W.F., Greyling, H.J., Lindsey, G.G., Retief, J.D., Rodrigues, J.D., Schwager, S.,
554 Sewell, B.T., 1989. Isolation and characterization of histones. Methods Enzymol. 170, 431-523.
555 Williamson, D.H., Brosnan, J., 1974. Concentrations of metabolites in animal tissues. In: (Bergmeyer, H.
556 Ed.) Methods of Enzymatic Analysis, 2nd Ed., vol. 4. Weinheim, Verlag Chemie; New York,
557 Academic Press, pp. 2266-2302.
558 Wu, C.W., Biggar, K.K., Storey, K.B., 2013. Dehydration mediated microRNA response in the African
559 clawed frog *Xenopus laevis*. Gene 529, 269-275.
560 Yang, T., Sauve, A.A., 2006. NAD metabolism and sirtuins: metabolic regulation of protein deacetylation in
561 stress and toxicity. AAPS J 8, E632-643.
562
563
564

565 **Figure legends**

566

567 Fig. 1. Amounts of sumoylated and acetylated proteins in liver homogenates of winter and summer bullfrog
568 tadpoles. Tissue homogenates (two of three samples/group) were analyzed by SDS-PAGE, followed by
569 Coomassie Brilliant Blue (*CBB*) staining, and western blotting using antibodies against *SUMO-1* and
570 acetylated lysine (*Kac*). Arrowheads denote bands whose intensities were different between the tadpoles
571 collected in winter and in summer. These experiments were repeated at least twice using the liver
572 homogenates from different animals in the same group, with similar results. Molecular markers used were
573 phosphorylase b (97 kDa), BSA (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin
574 inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa).

575

576 Fig. 2. Levels of epigenetic modification of core histones in liver homogenates of seasonally acclimatized
577 and thermally acclimated bullfrog tadpoles. Tissue homogenates (each $n = 8$) were prepared from the livers
578 of bullfrog tadpoles that were seasonally acclimatized (*A*, *winter* and *summer*) and acclimated to 4°C and
579 21°C for 3 days or for 2 weeks (*B*). Three of 8 homogenates/group were then analyzed by SDS-PAGE,
580 followed by western blotting using antibodies against acetylated histone H3 at lysine 9 (*H3K9ac*) and
581 histone H4 (*H4ac*), trimethylated histone H3 at lysine 9 (*H3K9me3*) and lysine 36, (*H3K36me3*). Band
582 intensities of modified histones were analyzed and are expressed relative to those of histone H3 pan (*H3*) or
583 H4 pan (*H4*). Each value represents mean ± SEM ($n = 3$). Differences between groups were analyzed by the
584 Student's *t*-test (panel *A*) or by a one-way ANOVA, with the Scheffe's or Fisher's test for multiple
585 comparisons (panel *B*). Asterisks denote significantly different means between two groups (*, $p < 0.05$; **,
586 $p < 0.01$). These experiments were repeated at least three times using the liver homogenates from different
587 animals ($n = 8$) in the same group, with similar results (Suppl. Figs. 1 and 2). To examine the effects of two
588 factors (temperature and experimental period) on the amount of H3K9ac in the four thermal acclimated

589 groups, we performed a two-way ANOVA (Suppl. Fig. 3).

590

591 Fig. 3. Transcript amounts of genes involved in histone acetylation and deacetylation in livers of seasonally
592 acclimatized and thermally acclimated bullfrog tadpoles. RNA was prepared from the liver of bullfrog
593 tadpoles (each $n = 8$) that were seasonally acclimatized (*A*, *winter* and *summer*) and acclimated to 4°C and
594 21°C for 3 days or for 2 weeks (*B*). The RNA samples were then analyzed by real-time reverse
595 transcription-quantitative polymerase chain reaction (RT-qPCR). Twelve genes were examined, divided
596 into the following three categories: (1) histone acetylation (6 genes; *crebbp*, *kat2a*, *ncoa1*, *ncoa2*, *ncoa3*
597 and *p300*), (2) histone deacetylation (3 genes; *hdac1*, *hdac3* and *sirt1*), (3) known cold- or heat-response
598 genes in vertebrates (*cirp*, *scd1* and *hsp90*). Full names of gene tested are shown in Supplementary Table 1.
599 The vertical axis represents the amount of gene transcripts after normalization to *actb*, and the values are
600 expressed relative to those of the winter or 4°C-acclimated animals that were set to 1.0. Each value
601 represents the mean ± SEM ($n = 8$). Differences between groups were analyzed by the Student's *t*-test
602 (panel *A*) or by a one-way ANOVA, with the Scheffe's test for multiple comparisons (panel *B*). Asterisks
603 denote significantly different means between two groups (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). These
604 experiments were repeated twice using the RNAs from different preparations from the same animals (each
605 $n = 8$) (Suppl. Fig. 4). To examine the effects of two factors (temperature and experimental period) on the
606 transcript amount of *crebbp* and *ncoa2* in the four thermal acclimated groups, we performed a two-way
607 ANOVA (Suppl. Fig. 5).

608

609 Fig. 4. Histone variant levels in liver homogenates of seasonally acclimatized and thermally acclimated
610 bullfrog tadpoles. Tissue homogenates (each $n = 8$) were prepared from the livers of bullfrog tadpoles that
611 were seasonally acclimatized (*A*, *winter* and *summer*) and acclimated to 4°C and 21°C for 3 days or for 2
612 weeks (*B*). Three of 8 homogenates/group were then analyzed by SDS-PAGE, followed by western blotting

using antibodies against histone H2A.Z, macroH2A and H3.3. Band intensities of variant histones were analyzed and expressed relative to those of histone H4 pan (*H4*). Each value represents the mean ± SEM (n = 3). Asterisks denote significantly different means between two groups (*, $p < 0.05$; **, $p < 0.01$). Differences between groups were analyzed by the Student's *t*-test (panel *A*) or by a one-way ANOVA, with the Scheffe's test for multiple comparisons (panel *B*). These experiments were repeated at least three times using the liver homogenates from different animals (n = 8) in the same groups, with similar results (Suppl. Figs. 6 and 7). To examine the effects of two factors (temperature and experimental period) on the amount of H2A.Z in the four thermal acclimated groups, we performed a two-way ANOVA (Suppl. Fig. 3).

Fig. 5. Transcript amounts of genes for histone variants in livers of seasonally acclimatized and thermally acclimated bullfrog tadpoles. RNA was prepared from the liver of bullfrog tadpoles (each n = 8) that were seasonally acclimatized (*A*, *winter* and *summer*) and acclimated to 4°C and 21°C for 3 days or for 2 weeks (*B*). The RNA samples were then analyzed by real-time reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Gene transcripts investigated were *h2afz*, *h2afv*, *h2afy*, *h2afy2* and *h3f3a.* Full names of gene tested are shown in Supplementary Table 1. The vertical axis represents the amount of gene transcripts after normalization to *actb*, and the values are expressed relative to those of the winter or 4°C-acclimated animals that were set to 1.0. Each value represents the mean ± SEM (n = 8). Differences between groups were analyzed by the Student's *t*-test (panel *A*) or by a one-way ANOVA, with the Scheffe's test for multiple comparisons (panel *B*). Asterisks denote significantly different means between two groups (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). These experiments were repeated twice using the RNAs from different preparations from the same animals (each n = 8) (Suppl. Fig. 8). To examine the effects of two factors (temperature and experimental period) on the transcript amount of *h2afz* in the four thermal acclimated groups, we performed a two-way ANOVA (Suppl. Fig. 5).

637

Table1. Morphological data of bullfrog tadpoles collected in different seasons and those acclimated to different temperatures for 3 days and 2 weeks.

	Seasonal acclimatization				Thermal acclimation							
	Winter		Summer		3 days				2 weeks			
	3–8°C		20-26°C		4°C		21°C		4°C		21°C	
Body length (cm)												
Start of experiment		-		-	9.8	± 0.3	9.1	± 0.3	9.4	± 0.2	9.1	± 0.3
End of experiment	8.6	± 0.2	8.7	± 0.2	10.0	± 0.3	9.2	± 0.3	9.3	± 0.3	9.0	± 0.2
Body weight (g)												
Start of experiment		-		-	8.6	± 0.8	7.7	± 0.8	7.3	± 0.6	7.7	± 0.7
End of experiment	6.4	± 0.4	6.3	± 0.3	8.3	± 0.7	7.4	± 0.8	7.4	± 0.7	7.4	± 0.6
Stage (TK)												
Start of experiment		-		-	8.4	± 0.5	8.2	± 0.7	7.9	± 0.6	7.8	± 0.7
End of experiment	7.3	± 0.4	8.9	± 0.8	8.8	± 0.5	8.3	± 0.8	8.1	± 0.7	8.6	± 0.6
Liver												
Wet weight (g)	0.20	± 0.02[a]	0.13	± 0.01[b]	0.26	± 0.05	0.20	± 0.03	0.23	± 0.03	0.22	± 0.01

Tadpoles were collected in winter (from December to January) and in winter (from July to September). After winter tadpoles were habituated in laboratory conditions to 4°C for 7 days, they were acclimated to 21°C and 4°C for 3 days and 2 weeks. Developmental stages of the tadpoles in each group ($n = 8$) were recorded according to the criteria of Taylor and Kollros (1946). Data were expressed as means ± SEM. Different letters denote significant different means between the winter and summer samples ($p < 0.05$).

638

639

640

Table 2. Contents of free coenzyme A (CoA-SH) and acetyl coenzyme A (acetyl-CoA) in the bullfrog tadpole liver.

Biological sample	CoA-SH			Acetyl-CoA			Acetyl-CoA/CoA-SH		
Tadpole liver									
Seasonal acclimatization									
Winter (3–8°C)	7.52	± 0.45	(5)	11.5	± 0.77 [a]	(5)	1.5	± 0.06 [a]	(5)
Summer (20–26°C)	9.77	± 1.88	(5)	7.33	± 0.91 [b]	(5)	0.9	± 0.20 [b]	(5)
Thermal acclimation									
3 days									
4°C	6.51	± 0.36 [a]	(5)	13.3	± 0.86 [a]	(5)	2.1	± 0.20	(5)
21°C	4.64	± 0.38 [b]	(5)	9.74	± 0.97 [b]	(5)	2.2	± 0.32	(5)
2 weeks									
4°C	5.42	± 0.44	(6)	4.01	± 0.83	(6)	0.8	± 0.16	(6)
21°C	4.94	± 0.11	(5)	4.64	± 0.79	(5)	0.9	± 0.15	(5)
Rat liver (acclimated to 22–24°C)	151.60	± 11.14	(5)	17.30	± 5.09	(5)	0.13	± 0.05	(5)

Data were expressed as means ± SEM (n = 5–6) (nmol/g wet weight). Different letters denote significantly different means between two groups (winter vs. summer, and 4°C vs. 21°C) ($p < 0.05$).

641

Fig. 1

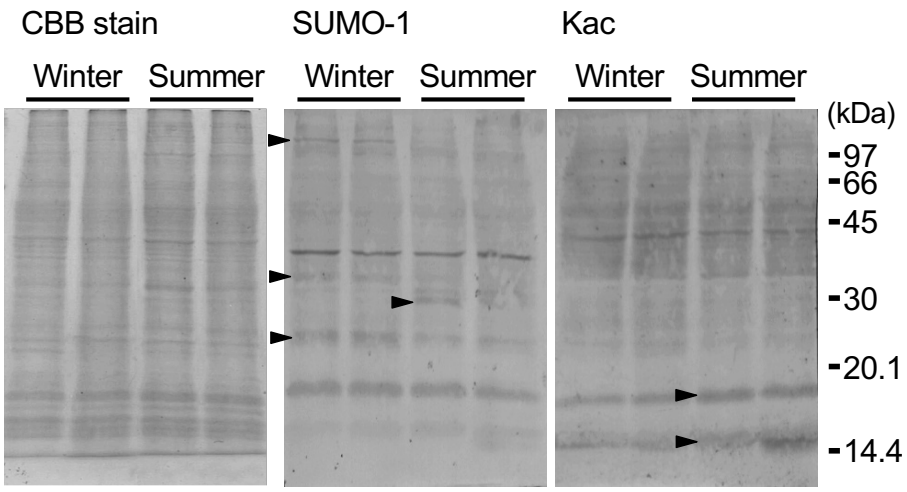

CBB stain
SUMO-1
Kac
Winter
Summer
Winter
Summer
Winter
Summer
(kDa)
97
66
45
30
20.1
14.4

Fig. 2

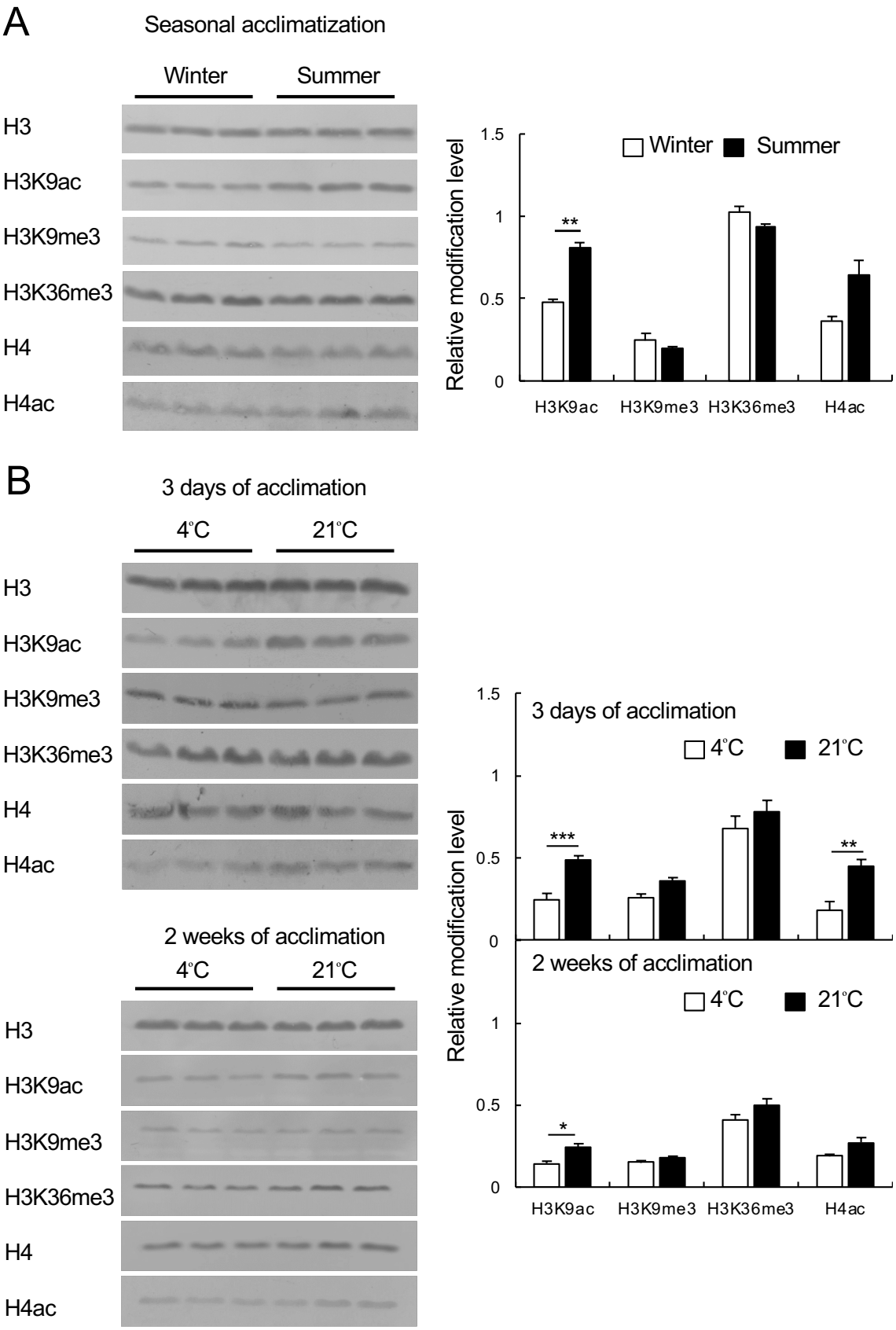

A
Seasonal acclimatization
Winter
Summer
H3
H3K9ac
H3K9me3
H3K36me3
H4
H4ac
Relative modification level
1.5
1
0.5
0
Winter
Summer
**
H3K9ac
H3K9me3
H3K36me3
H4ac
B
3 days of acclimation
4°C
21°C
H3
H3K9ac
H3K9me3
H3K36me3
H4
H4ac
2 weeks of acclimation
4°C
21°C
H3
H3K9ac
H3K9me3
H3K36me3
H4
H4ac
Relative modification level
3 days of acclimation
4°C
21°C
***
**
2 weeks of acclimation
4°C
21°C
*
H3K9ac
H3K9me3
H3K36me3
H4ac

Fig. 3

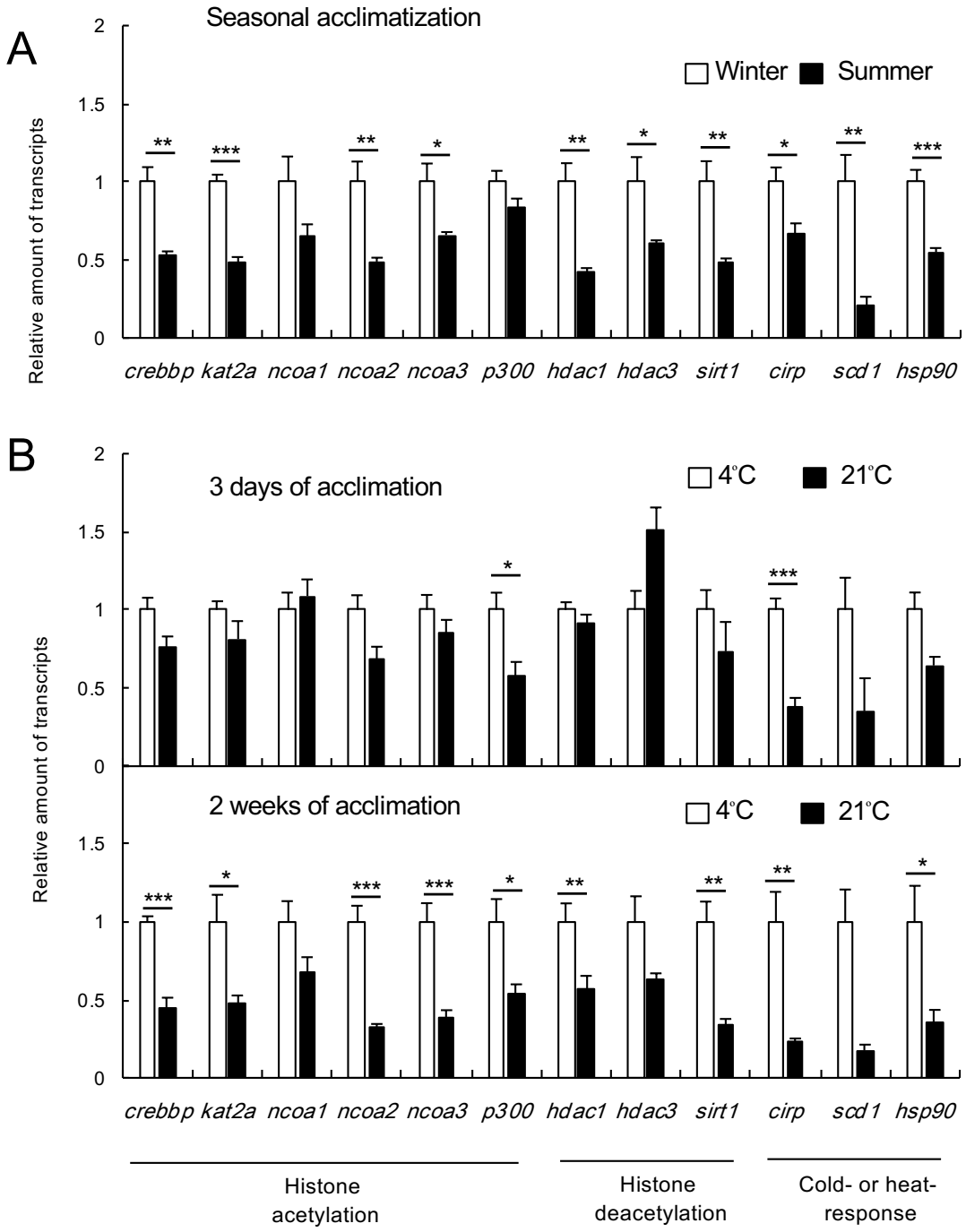

A
Seasonal acclimatization
Winter
Summer
Relative amount of transcripts
2
1.5
1
0.5
0
crebbp kat2a ncoa1 ncoa2 ncoa3 p300 hdac1 hdac3 sirt1 cirp scd1 hsp90
B
3 days of acclimation
4°C
21°C
2 weeks of acclimation
4°C
21°C
Relative amount of transcripts
crebbp kat2a ncoa1 ncoa2 ncoa3 p300 hdac1 hdac3 sirt1 cirp scd1 hsp90
Histone acetylation
Histone deacetylation
Cold- or heat-response

Fig. 4

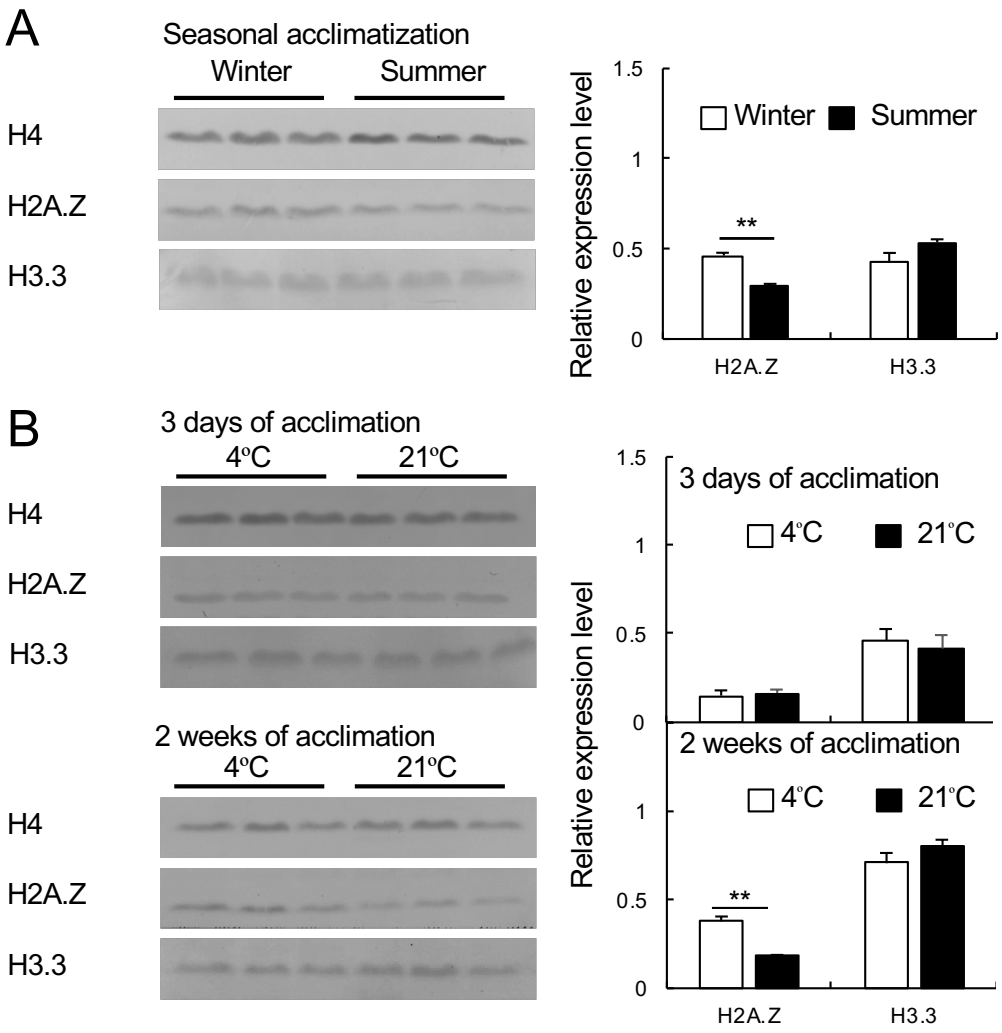
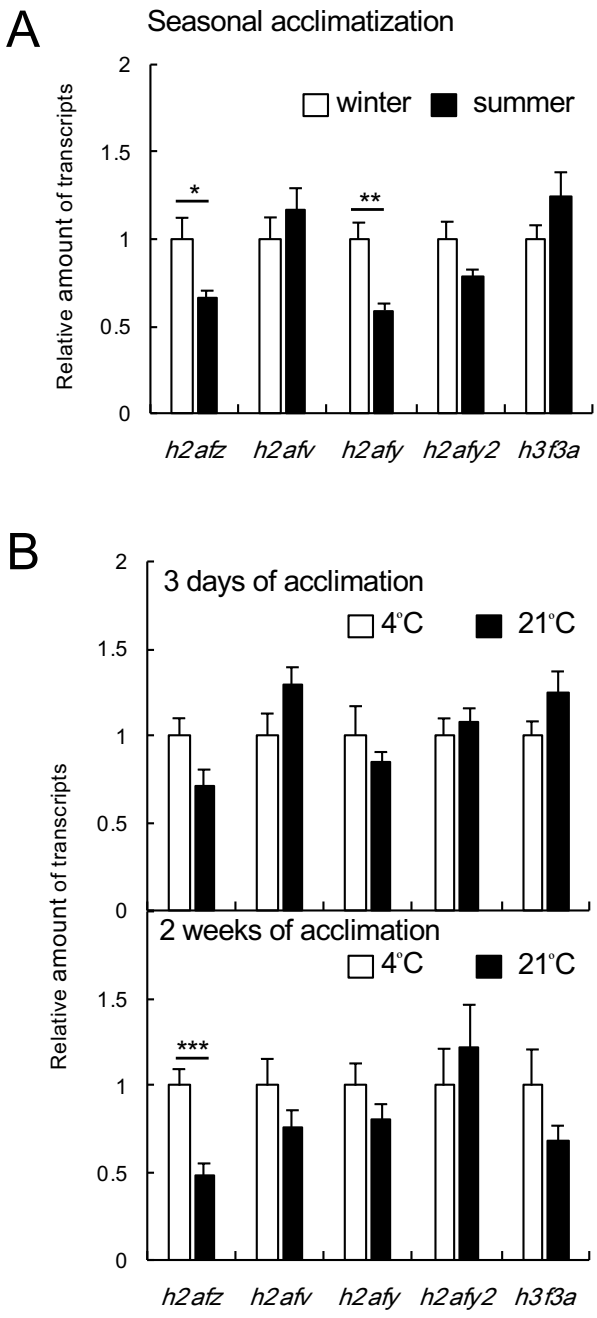

A
Seasonal acclimatization
Winter
Summer
H4
H2A.Z
H3.3
Relative expression level
1.5
1
0.5
0
Winter
Summer
**
H2A.Z
H3.3
B
3 days of acclimation
4°C
21°C
H4
H2A.Z
H3.3
2 weeks of acclimation
4°C
21°C
H4
H2A.Z
H3.3
Relative expression level
1.5
1
0.5
0
3 days of acclimation
4°C
21°C
2 weeks of acclimation
4°C
21°C
**
H2A.Z
H3.3

Fig. 5


A
Seasonal acclimatization
winter
summer
Relative amount of transcripts
2
1.5
1
0.5
0
*
**
h2afz
h2afv
h2afy
h2afy2
h3f3a
B
3 days of acclimation
4°C
21°C
2 weeks of acclimation
4°C
21°C
***
Relative amount of transcripts
h2afz
h2afv
h2afy
h2afy2
h3f3a