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# Analysis of global and gene-specific acetylation of histones in the liver of American bullfrog (*Rana catesbeiana*) tadpoles acclimated to low temperature

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Abbreviations: acetyl-CoA, acetyl coenzyme A; ChIP, chromatin immunoprecipitation; CoA-SH, free coenzyme A; cyp7a1, cytochrome P450 7a1; H3K9me3, trimethylated histone H3 at lysine 9; H3K36me2, dimethylated histone H3 at lysine 36; 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; PAGE, polyacrylamide gel electrophoresis; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; scd, stearoyl-CoA desaturase; SDS, sodium dodecyl sulfate; SEM, standard error of the mean; TBS, Tris-buffered saline; TH, thyroid hormone.

**Abstract (235 words)**

Severe environmental stressors such as low temperatures can affect gene expression by changing epigenetic states. American bullfrog (*Rana catesbeiana*) can overwinter as tadpoles, which can be active even in winter. However, the molecular mechanisms of epigenetic controls by which the tadpoles acclimate to low temperature are still unclear. In this study, we aimed to clarify the molecular mechanisms of global and gene-specific epigenetic regulations of low-temperature acclimation. We found that the global acetylation was decreased in the liver of bullfrog tadpoles acclimated to low temperature. The amounts of transcripts for two histone acetyltransferases were higher in the liver of tadpoles acclimated to low temperature than in those acclimated to warm temperature, while we observed no significant differences in the amounts of transcripts for histone deacetylases. We also found that the amounts of transcripts and acetylated histones on the specific temperature-responsive genes *scd* and *cyp7a1* whose transcripts were increased and decreased, respectively, in response to low temperature were positively correlated. Cellular acetyl-CoA levels were higher in the liver of tadpoles acclimated to low temperature than in those acclimated to warm temperature. These results contradicted the states of histone acetylation, suggesting that bullfrog tadpoles have different epigenetic mechanisms to modify the histones when compared with those of other organisms such as reptiles and mammals, even though the relationship between the transcript amount and the states of histone acetylation on temperature-responsive genes was similar to that of mammals.

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

## 1. Introduction

American bullfrog (*Rana catesbeiana*) is a eurythermal animal native of the eastern United States and is now widely introduced in many parts of North America, the Caribbean region, South America, Asia, and Europe (Kahrs, 2006; Orchard, 2011; Schloegel et al., 2010; Snow and Witmer, 2010). Adult bullfrogs breed in June and July in Kentucky, and hatched tadpoles grow until October (Viparina and Just, 1975). The bullfrog tadpoles that overwinter resume growth in early May and metamorphose to froglets (Viparina and Just, 1975). Overwintering green frog (*Lithobates clamitans*) tadpoles are active even in winter and maintain swimming performance (Gray et al., 2016). Metamorphosis is known to be controlled by thyroid hormone (TH), but under low-temperature conditions (5 °C), metamorphosis does not proceed even in the presence of TH (Frieden, 1967). Nuclear receptors functioning as transcription factors control the actions of TH (Mangelsdorf et al., 1995), but the expression of TH-responsive genes controlled by them does not change at low temperatures in the liver of bullfrog tadpoles (Mochizuki et al., 2012; Murata and Yamauchi, 2005). These facts suggest that temperature may be upstream of TH in the TH signaling pathway. However, details of the molecular mechanisms by which metamorphosis stops at low temperature have not been clarified. Mochizuki et al. (2012) revealed that histone modifications like di or trimethylated histone H3 at lysine 36 (H3K36me2 or H3K36me3, respectively) caused by TH treatment were inhibited by low temperature in the gene region of TH responsive genes. This result suggests that there is a temperature-sensitive step in the histone modification process caused by nuclear receptor cofactors.

Ectothermic organisms like fish can adapt to a low temperature by changing the transcript amounts and the activity of enzymes relating to energy metabolism such as lipid metabolism (Chen et al., 2018; Trueman et al., 2000). Gracey et al. (2004) reported that 260 transcripts of common carp were significantly and differentially expressed by cold exposure in all tissues tested in their study, including liver, and the transcript amounts for the majority (252) of these transcripts were increased. We also found that, during cold exposure, the transcript amount for the majority of genes related to energy metabolism including stearoyl-CoA desaturase (*scd*) was higher, while that for cholesterol-metabolizing gene cytochrome P450 7a1 (*cyp7a1*) was lower, in the liver of cold-acclimated tadpoles (Suzuki et al., 2016). However, it is still unclear how such genome-wide expression is controlled in

the liver of lower vertebrates at low temperatures. One possible endpoint is epigenetic regulation including histone modifications, DNA methylation and expression of microRNA (Storey and Storey, 2017). There are several reports in which authors clarified that severe environmental stressors affected gene expression by changing epigenetic states, such as histone modifications and composition of specific histone variants. Krivoruchko and Storey (2010) reported that anoxia reduced the acetylation of histone H3 at lysine 9 (H3K9ac) by increasing the amounts of transcript and protein for several histone deacetylases (HDACs) and the activity of HDACs in the liver of red-eared slider turtles. The level of acetylated histones in the cells depends on the functions of HDACs and histone acetyltransferases (HATs), which transfer the acetyl group from acetyl coenzyme A (acetyl-CoA) to histones. In higher vertebrates, it is thought that the amounts of cellular acetyl-CoA, acetylated histones, and transcripts represent similar patterns (Carrer et al., 2017; Etchegaray et al., 2003). In addition, nuclear amounts of H2A variants H2A.Z and macroH2A were higher in the liver of eurythermal fish carp collected in winter than that collected in summer (Pinto et al., 2005; Simonet et al., 2013). However, it is not clear whether such epigenetic controls play an important role in the adaptation of amphibian species to low temperature.

In this study, we aimed to clarify the molecular mechanisms of how low temperature affects the global and gene-specific epigenetic states in the liver of bullfrog tadpoles because the liver plays an important role in energy metabolism. Therefore, we examined the acetylation levels of histones, transcript amounts of HATs and HDACs, and cellular free coenzyme A (CoA-SH) and acetyl-CoA levels in the liver of bullfrog tadpoles collected in summer and winter and of bullfrog tadpoles which were experimentally acclimated to low temperature. Finally, we also examined the effects of low temperature on histone modification levels in specific gene regions.

## 2. Materials and methods

### *2.1. Reagents*

CoA-SH, 3-Aminobenzoic acid ethyl ester and phenylmethylsulfonyl fluoride were obtained from Sigma-Aldrich (St. Louis, MO, USA). Protease inhibitors leupeptin, E-64, acetyl-CoA, and sulfosalicylic acid were purchased from Wako (Osaka, Japan). TaqMan reverse transcription reagents kit and Thunderbird SYBR qPCR mix were purchased from Applied Biosystems (Foster City, CA, USA) and Toyobo (Osaka, Japan), respectively. ProtoBlot AP System and alkaline phosphatase conjugated anti-rabbit immunoglobulin raised in goat (S3731, used as secondary antibody for western blotting) were purchased from Promega (Madison, WI, USA). Rabbit polyclonal antibodies against H3K36me3 (ab9050) were obtained from Abcam (Tokyo, Japan), and those against trimethylated histone H3 at lysine 9 (H3K9me3; 07-442), H3K9ac (17-615), and acetylated histone H4 that recognizes acetylated lysines 5, 8, 12, and 16 (H4ac; 06-598), histone H3 pan (07-690), and histone H4 pan (05-858) were obtained from Merck Millipore (Darmstadt, Germany). All other reagents used in this study were of the highest grade commercially available.

### *2.2. Animal care and experimental design*

American bullfrog tadpoles (5.7–10.9 g) were collected in summer (in June, 2016 and September, 2017; average water temperature, 22–30 °C; daylight hours, 13 h 29 min) and in winter (in January 2017; average water temperature, 5–14 °C; daylight hours, 10 h 01 min) from ponds in Shizuoka or in Ibaraki through a commercial supplier in Japan. The developmental stages of the tadpoles were determined according to the criteria for *Rana pipiens* (Taylor and Kollros, 1946). Tadpoles (each $n$ = 8–15) collected in summer (in June 2016 and September 2017) and winter (in January 2017) were anesthetized by immersion in 0.02 % 3-aminobenzoic acid ethyl ester, without acclimation to laboratory conditions. After measuring their body weight and body length, their liver was 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 cold acclimation experiments, only tadpoles collected in summer were used. They were maintained in aerated and dechlorinated tap water at 22 °C, under natural lighting conditions, and

were fed ad libitum boiled spinach (approximately 0.5 g of a frozen block/tadpole) at 9:00 AM three times per week. After acclimation to laboratory conditions at 22 °C for 1 week, 32 tadpoles were divided into four groups (8 individuals/10 L water per group): two 22 °C and two 4 °C groups. For 22 °C groups, tadpoles were maintained at 22 °C until Day 3 or Day 30. For 4 °C groups, tadpoles were subjected to a stepped cooling regime of 1 °C/2 h to a maximum of 6 °C/day, to 4 °C, over 4 days (from Day -3 to Day 0), and then maintained at 4 °C until Day 3 or Day 30. The mean body weight of each group was adjusted to be similar at the beginning of the experiment. Half of the water volume (5 L) in the aquaria was changed 3 times per week on the next day after feeding. On Day 3 and Day 30, body weight, body length and developmental stages of tadpoles were measured. Tadpoles were then anesthetized with 0.02 % 3-aminobenzoic acid ethyl ester, and the liver was collected and stored at -84 °C.

All housing and experimental procedures were conducted in accordance with the guidelines for the care and use of laboratory animals of Shizuoka University (permit #29F-8) under the international guideline “Act on Welfare and Management of Animals” (Ministry of Environment of Japan).

*2.3. Preparation of liver homogenates and western blotting*

Preparation of liver homogenates and western blotting were performed by previously described protocols (Ishihara et al., 2019). In brief, frozen liver (approximately 20 mg) was homogenized in 500 μL of homogenization buffer (50 mM Tris-HCl, pH 7.5, 25 mM KCl, 250 mM sucrose, 10 mM sodium butyrate, 1 mM sodium orthovanadate, 0.5 mM phenylmethylsulfonyl fluoride, 2 mM leupeptin, 1.4 mM E-64) (Rumbaugh and Miller, 2011) on ice, with a Polytron homogenizer. Homogenate was immediately mixed with 2× sodium dodecyl sulfate (SDS) gel-loading buffer (140 mM Tris-HCl, pH 6.8, 22.4 % glycerol, 6 % SDS, 0.02 % bromophenol blue, 10 % mercaptoethanol). The mixture was boiled for 5 min and then stored at -20 °C for later use.

For western blotting, homogenates containing 20–50 μg proteins were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) with resolving gel containing 15 % acrylamide. After electrophoresis, the resolved proteins were transferred onto a polyvinylidene difluoride membrane

(0.22 μm, FluoroTrans®; PALL, Port Washington, New York, USA). 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 incubated for 1 h at room temperature with rabbit primary antibody directed against H3K36me3 (1:1000), H3K9me3 (1:500), H3K9ac (1:20000), H4ac (1:500), H3 pan (1:25000) and H4 pan (1:30000) in 1 % skim milk in TBS. The membrane was rinsed three times with TBS containing 0.1 % Tween 20 and then incubated with the secondary antibody (1:2500) in 1 % skim milk in TBS for 30 min at room temperature. Immunoblot was developed using a detection kit containing 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium to 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 loading in western blots, the intensity of bands stained with antibodies directed against the modified histones in each lane was normalized against bands stained with antibodies against the histones H3 pan or H4 pan. The protein content of the homogenates was determined by the Lowry method (Jain et al., 2002) using bovine serum albumin as standard.

*2.4. Preparation of liver total RNA and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis*

Preparation of liver total RNA and RT-qPCR were performed following previously described protocol (Ishihara et al., 2019). In brief, total RNA was extracted from liver (~0.1 g) by the acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). 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 *R. catesbeiana* genes were obtained from public databases. Detailed information of primer sets is shown in Supplementary Table 1. The expression of genes of interest was estimated in triplicate using Thunderbird SYBR qPCR mix and Thermal Cycler Dice RealTime System Single TP850 (TaKaRa, Shiga, Japan) 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 s and 60 °C for 1 min, as previously described (Tamaoki et al., 2016). All assays gave unique dissociation curves. PCR

efficiency 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, and listed in Supplementary Table 1. 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 summer or 22 °C-acclimated group. 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 were the most invariable among the experimental groups, we selected *actb* as a reference gene.

*2.5. Extraction of CoA compounds and high-performance liquid chromatography (HPLC) analysis of acetyl-CoA and CoA-SH*

Extraction of CoA compounds and HPLC analysis were performed by following the protocol previously described (Ishihara et al., 2019). In brief, 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 centrifuged at 600 *g* for 10 min, and the supernatant was filtered through a disc filter (0.45 m, 03CP045AN, Advantec; Tokyo, Japan) and 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 $\varepsilon_{260}$ for CoA-SH (14.6) and $\varepsilon_{259}$ for acetyl-CoA (15.4) (King et al., 1988). The HPLC system consisted of a pump (model 600), a controller (model 600), a dual absorbance detector (model 2487), and an autosampler (model 717 plus), with Empower software, from Waters (Milford, MA, USA). An aliquot (20 μL) of the tissue extract was injected onto a reverse-phase C18 analytical column (Mightysil RP-18 GP, 4.6 × 250 mm, 5 μm particle diameter, Kanto Chemical; Tokyo, Japan), equipped with a guard column (Mightysil RP-18 GP, 4.6 × 5 mm, 5 μm) of the same packing material. The column temperature was kept at 40 °C. The samples were kept at 4 °C inside a closed chamber of the autosampler. Solvent A consisted of 100 mM sodium dihydrogen phosphate and 75 mM sodium acetate, pH 4.6. Solvent B was a mixture of Solvent A and methanol (7/3, v/v). The starting mobile phase (Solvent A/Solvent 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 min and then 100 %

Solvent B was applied for the subsequent 20 min. This was followed by a linear gradient back to the starting mobile phase over 3 min. The column was re-equilibrated with the starting mobile phase for 15 min before the start of the next run. Each extracted sample and the authentic standards (10 pmol) were injected two times, and eluted compounds were monitored by the absorbance at 254 nm. Peaks for CoA-SH and acetyl-CoA were identified by comparison of retention times with those of authentic standards determined on the same day. The retention times for CoA-SH and acetyl-CoA and their day-to-day variations were 17.69 ± 0.02 ($n$ = 8) min for CoA-SH and 21.03 ± 0.02 ($n$ = 8) min for acetyl-CoA. CoA compounds in the liver extracts were quantified using the linear calibration curves [y = 0.7747x - 0.5711, $r^2$ = 0.9999, for CoA-SH ranging from 2.5 to 300 pmol; and y = 0.7369x - 0.2954, $r^2$ = 0.9997, for acetyl-CoA ranging from 2.5 to 300 pmol. y, peak area (μVsec × $10^{-3}$); x, amount of CoA 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 groups were analyzed by a Student's *t*-test. Differences were considered significant at $p < 0.05$.

## 3. Results

### *3.1. Morphological parameters of seasonally acclimatized or thermally acclimated tadpoles*

Although body length and body weight were significantly higher in tadpoles collected in winter than in those collected in summer, this did not happen between condition factors (CFs), which seemed to be similar (Table 1). Tadpoles collected in winter were at an earlier developmental stage than those in summer. Liver weight and hepatosomatic index (HSI) of tadpoles collected in winter were higher than those collected in summer. Although the liver weight of tadpoles thermally acclimated to low temperature (4 °C) for 3 days and 30 days was significantly higher than that of tadpoles acclimated to 22 °C, we found no differences in HSIs between 4 °C and 22 °C (Table 1).

### *3.2. Changes of global histone modifications in the liver of tadpoles thermally acclimated to low temperature for 3 days or 30 days*

We found that the amounts of acetylated histones H3K9ac and H4ac in the liver of tadpoles thermally acclimated to low temperature (4 °C) for 3 days and 30 days were significantly lower than of tadpoles acclimated to 22 °C (Fig. 1). We observed no significant differences in the amount of methylated histone H3, H3K9me3, and H3K36me3 between the livers of tadpoles acclimated to 4 °C and 22 °C (Fig. 1).

### *3.3. Amount of gene transcripts related to histone modifications in the liver of cold-acclimated tadpoles*

To clarify the molecular mechanisms for the changes in histone acetylation, we examined the amounts of gene transcripts for histone-modifying enzymes with temperature-responsive proteins in the liver of tadpoles acclimated to low temperature. The amounts of transcripts for cold-induced genes (*cirp* and *scd*) were significantly higher, and the amounts of transcript for *cyp7a1* were significantly lower in the liver of cold-acclimated (4 °C) than in warm-acclimated tadpoles (22 °C) for 3 days and 30 days (Fig. 2). The transcript amounts for several HAT genes were significantly higher in the liver of cold tadpoles for 3 days (*ncoa2* and *p300*; Fig. 2A) and 30 days (*crebbp*, *ncoa2* and *p300*; Fig. 2B).

There were no significant differences in gene expression levels of HDACs between the 22 °C group and 4 °C group for 3 days and 30 days (Fig. 2A and Fig. 2B, respectively).

*3.4. Cellular CoA-SH and acetyl-CoA contents in the liver of cold-acclimated tadpoles*

As we could detect similar results in histone modification and gene expression between 3 day- and 30 day-acclimation to low temperature, we examined the cellular contents of CoA-SH and acetyl-CoA in the liver of cold-acclimated tadpoles only for 3 days and those acclimatized to summer and winter. Acetyl-CoA level and the ratio of acetyl-CoA/CoA-SH were significantly higher in the winter tadpole liver than in the summer one (Table 2). Acetyl-CoA level and the ratio of acetyl-CoA/CoA-SH were significantly higher in the liver of tadpoles acclimated to 4 °C than those acclimated to 22 °C (Table 2). There were no significant differences in CoA-SH content in the livers of seasonally acclimatized and thermally acclimated tadpoles (Table 2).

*3.5. Changes of histone acetylation on specific gene regions in the liver of cold-acclimated tadpoles*

To clarify the changes of histone acetylation on specific gene regions in the liver of cold-acclimated tadpoles, we performed chromatin immunoprecipitation (ChIP) assay. Thus, we examined *scd* or *cyp7a1* gene regions whose transcript amounts were significantly higher or lower in the liver of cold-acclimated tadpoles (Fig. 2). The amount of H3K9ac on the cold-induced gene *scd* was significantly higher, while that on the cold-suppressed gene *cyp7a1* was significantly lower in the liver of tadpoles acclimated to low temperature (4 °C) for 3 days than that acclimated to 28 °C (Fig. 3). We did not observe significant differences in the normal IgG levels on both gene regions (Fig. 3).

## 4. Discussion

In the present study, we found similar CFs and different HSIs between the tadpoles collected in winter and summer. We also found that global histone acetylation was low in the liver of bullfrog tadpoles collected in winter or those acclimated to low temperature, and the histone acetylation states could not be explained by the amount of transcripts for histone-modifying enzymes.

CFs were similar between the tadpoles collected in winter and summer, while HSI in the tadpoles collected in winter was higher than that of tadpoles collected in summer, suggesting hypertrophy and/or hyperplasia of liver in the tadpoles collected in winter. Overwintering green frog tadpoles are active in winter (Gray et al., 2016), which may result in the simple growth of tadpoles during winter and similar CFs between the tadpoles collected in winter and summer. It is also known that wild juvenile bullfrogs increased their glycogen stores in liver prior to overwintering (Farrar and Dupre, 1983). We examined the glycogen contents in the liver of tadpoles collected in winter and summer and found that stored glycogen was increased in the liver of tadpoles seasonally acclimatized to low temperature (winter, data not shown). This result raises a possibility that increased HSI in the tadpoles collected in winter may be due to hypertrophy of the liver which accumulates glycogen.

The amounts of H3K9ac and H4ac were lower in the liver of cold-acclimated tadpoles than in the liver of tadpoles acclimated to 22 °C (Fig. 1). Similar results were observed in the liver of tadpoles acclimatized to low temperature (in the liver of tadpoles collected in winter) (Ishihara et al., 2019). These results suggest that deacetylation or acetylation of H3K9ac and K4ac had been proceeded or inhibited, respectively, in the liver of cold-acclimated tadpoles. However, we could not observe significant differences in transcript amounts of any HDACs tested between the 22 °C group and cold-acclimated groups (Fig. 2), while transcript amounts of all HDACs tested were higher in the liver of tadpoles collected in winter than in the liver of those collected in summer (Ishihara et al., 2019). In contrast, the transcript amounts of HAT enzymes (*ncoa2* and *p300* in 3 and 30 days of acclimation and *crebbp* only in 3 days acclimation) were higher in the liver of tadpoles acclimated to low temperature than in the liver of those acclimated to 22 °C (Fig. 2), and similar results were observed in the liver of tadpoles seasonally acclimatized to low temperature (Ishihara et al., 2019). The transcript amounts for HATs and HDACs were inconsistent with the levels of histone acetylation (Fig.

1), while those for only HDACs were consistent with the levels of histone acetylation in the liver of seasonally acclimatized tadpoles to low temperature (Ishihara et al., 2019). Our recent research has revealed that sirtuins, $NAD^+$-dependent HDACs, were active at least at 21 °C (Ishihara et al., 2019). Thus, we concluded that the changes of histone acetylation states could not be explained by the transcript amounts of sirtuins and/or the activity of sirtuins in the liver of tadpoles acclimated to low temperature. There are several reports in which authors clarified the relationship between the protein amounts of histone-modifying enzymes and the states of histone modification. In a study using the liver of freshwater turtles, Krivoruchko and Storey (Krivoruchko and Storey, 2010) revealed that exposure to 5 h anoxia increased the transcript and protein amounts for HDAC1 and HDAC4 and decreased the acetylation of histone H3 at lysines 9 and 23. Moreover, the exposure of the turtles to anoxia increased the protein amounts of histone-methylating enzymes such as Set1/Ash2 histone methyltransferase complex subunit ASH2, retinoblastoma-binding protein 5 and euchromatic histone-lysine N-methyltransferase 2, and the methylation of H3K4 and H3K9 were upregulated (Wijenayake et al., 2018). By using freezing tolerant frogs, Hawkins and Storey (2018) reported that the protein amounts of SMYD2, a histone methyltransferase, and the amounts of H3K4me1 were lower in freezing conditions (-4.0 °C for 45 min and then -2.5 °C for 24 h) than in control (5 °C). These results indicate that the protein levels of the histone-modifying enzymes correlate with the histone modification states in at least these two species. In this study, because only the amounts of the transcripts were examined, it will be necessary to investigate the amounts of proteins for HAT or HDAC enzymes.

In this study, we found a negative correlation between the amounts of acetyl-CoA and acetylated histone H4 that was different from positive correlation observed in mammals. The amounts of acetyl-CoA were higher in the livers of the cold groups (winter and 4 °C-acclimated group; Table 2) than those of the warm groups (summer and 22 °C-acclimated group; Table 2). These results were inconsistent with the histone acetylation level. It was reported that the treatment of acetyl-CoA to mammalian cell increased the amount of acetylated histone H3 in a dose-dependent manner (Mariño et al., 2014). This suggests that the amount of acetyl-CoA and histone acetylation may show a positive correlation in mammals, which is different from the bullfrog tadpoles found in this study. The

nucleocytoplasmic pool of acetyl-CoA is affected by several biological processes such as glycolysis, Krebs cycle, gluconeogenesis, fatty acid synthesis, beta-oxidation of fatty acids, and metabolism of acetate. In mammalian cells, ATP-citrate lyase catalyzes the production of acetyl-CoA, which is the major source for histone acetylation from citrate derived from glycolysis (Wellen et al., 2009). Recently, it is reported that lipid-derived acetyl-CoA is used as a major source of acetate for histone acetylation (McDonnell et al., 2016). Cold stress increased the transcript and/or protein amounts of gluconeogenesis-related genes like glucose-6-phosphatase and phosphoenolpyruvate carboxykinase in rat and bullfrog liver (Sepa-Kishi et al., 2018; Suzuki et al., 2016) and activity of lipolysis-related enzyme carnitine palmitoyltransferase (Suzuki et al., 2016) and decreased the transcript amounts of lipogenesis-related genes like acetyl-CoA carboxylase and fatty acid synthetase in rat and chicken liver (Nguyen et al., 2015; Sepa-Kishi et al., 2018). In addition, juvenile bullfrogs are known to reduce the hepatic lipid prior to overwintering (Farrar and Dupre, 1983). These results raise the possibility that lipolysis is induced and lipid-derived acetyl-CoA is increased in the liver of bullfrog tadpoles acclimated to low temperature, which could explain the increased amount of cellular acetyl-CoA observed in this study. The reason why the amount of acetylated histone is reduced despite the increased amount of acetyl-CoA may be that acetyl-CoA is produced not only from glycolysis and fatty acid beta-oxidation, but also from citric acid and acetic acid by ATP-citrate lyase and acyl-CoA synthetase short-chain family member 2, respectively. It is considered that the cellular compartmentalization of acetyl-CoA may be regulated by the localization of these enzymes. To clarify how the nucleocytoplasmic pool of acetyl-CoA is controlled and how the acetylation level of histones is controlled in the liver of bullfrog acclimated to low temperature, further studies on the amount and/or activity of enzymes relating to histone modifications and/or the synthesis, metabolism, and translocation of acetyl-CoA are required.

Finally, we examined the histone acetylation states in the gene regions of *scd* and *cyp7a1*, whose transcript amounts were increased and decreased by cold acclimation, respectively. As a result, it was revealed that the histone acetylation states and the amount of the transcripts showed a positive correlation (Fig. 3). Our results are consistent with the mammalian case (Etchegaray et al., 2003; Gao et al., 2016), suggesting that the chromatin structure is loose in the region where transcription is

activated. It will be necessary to investigate whether amphibians have different transcription regulatory mechanisms for gene expression responsive to different environmental stimuli such as fasting and low temperature.

In conclusion, we found that the global acetylation in the liver of bullfrog tadpoles acclimated to cold conditions was reduced. However, in a specific gene region, we observed a positive correlation between the amounts of the transcript and acetylated histone. To clarify the relationship between genome-wide expression and genome-wide states of histone modification for environmental stimuli response, further studies, using ChIP-seq analyses for example, are necessary.

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**Figure Legends**

Fig. 1. Histone modification levels in the liver of bullfrog tadpoles acclimated to 4 °C and 22 °C. Tissue homogenates (three samples/each group) were prepared from the livers of bullfrog tadpoles acclimated to low temperature for 3 days (*A*) and 30 days (*B*). The homogenates were analyzed by SDS-PAGE, followed by western blotting with antibodies against acetylated histone H3 at lysine 9 (*H3K9ac*), and histone H4 (*H4ac*), trimethylated histone H3 at lysine 9 (*H3K9me3*) and lysine 36 (*H3K36me3*). Band intensities of modified histones were analyzed and expressed relative to those of histone H3 pan (*H3*) and H4 pan (*H4*). Each value is represented as the mean ± SEM ($n = 3$). Asterisks denote significant differences between 4 °C and 22 °C (*, $p < 0,05$; ***, $p < 0.001$). These experiments were repeated at least twice using the liver homogenates from different tadpoles in the same group, with similar results.

Fig. 2. The amount of transcript for genes involved in histone acetylation and deacetylation in the livers of bullfrog tadpoles acclimated to 4 °C and 22 °C. Total RNAs were prepared from the liver of bullfrog tadpoles acclimated to low temperature for 3 days (*A*) and 30 days (*B*). The RNAs were analyzed by real-time reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The genes tested were categorized into three groups: 1) histone acetyltransferases (*crebbp*, *kat2a*, *ncoa1*, *ncoa2*, *ncoa3* and *p300*), 2) histone deacetylases (*hdac1*, *hdac3* and *sirt*), 3) temperature-responsive genes (*cirp*, *scd* and *cyp7a1*). Full names of genes are shown in Supplementary Table 1. The vertical axis represents the amount of transcripts after normalization with *actb*, and the values are expressed relative to those of the 22 °C-acclimated animals that were set to 1.0. Each value is the mean ± SEM ($n = 8$). Asterisks denote significant differences between two groups (*, $p < 0,05$; **, $p < 0,01$; ***, $p < 0.001$).

Fig. 3. Histone acetylation of in the liver of bullfrog tadpoles acclimated to low temperature. Acetylation of histone H3 at lysine 9 of *cyp7a1* and *scd* genes in the liver of bullfrog tadpoles acclimated to low temperature were examined. Signals of ChIP on *cyp7a1* and *scd* were detected by qPCR ($n = 8$). Each signal was converted to signal percentage for DNA input. These experiments

were repeated at least twice, with similar results. Antibody against normal IgG was used as negative control to examine the background levels. Asterisks denote significant differences between two groups (**, $p < 0,01$; ***, $p < 0.001$).

Table1. Morphological data of bullfrog tadpoles collected in different seasons and those acclimated to different temperatures for 3 and 30 days.

| | Seasonal acclimatization | | Thermal acclimation | | | |
|---|---|---|---|---|---|---|
| | Summer | Winter | 3 days | | 30 days | |
| | 22–30 °C | 5–14 °C | 22 °C | 4 °C | 22 °C | 4 °C |
| Body length (cm) | | | | | | |
| Start of experiment | - | - | 9.0 ± 0.1 | 8.8 ± 0.2 | 8.6 ± 0.1 | 8.9 ± 0.2 |
| End of experiment | 8.2 ± 0.2[a] | 10.1 ± 0.3[b] | 8.5 ± 0.2 | 8.8 ± 0.2 | 7.6 ± 0.4 | 7.0 ± 0.4 |
| Body weight (g) | | | | | | |
| Start of experiment | - | - | 6.6 ± 0.2 | 7.0 ± 0.4 | 6.6 ± 0.3 | 6.5 ± 0.2 |
| End of experiment | 5.7 ± 0.4[a] | 10.9 ± 1.1[b] | 6.9 ± 0.2 | 7.1 ± 0.4 | 5.0 ± 0.2 | 5.4 ± 0.3 |
| Stage (TK) | | | | | | |
| Start of experiment | - | - | 14.2 ± 0.2 | 13.9 ± 0.2 | 4.0 ± 0.5 | 4.1 ± 0.5 |
| End of experiment | 12.4 ± 0.3[a] | 8.3 ± 0.3[b] | 13.7 ± 0.2 | 13.4 ± 0.2 | 11.3 ± 1.4[a] | 6.8 ± 0.5[b] |
| Liver | | | | | | |
| Wet weight (g) | 0.16 ± 0.01[a] | 0.68 ± 0.10[b] | 0.16 ± 0.01[a] | 0.21 ± 0.02[b] | 0.09 ± 0.01[a] | 0.12 ± 0.00[b] |
| Condition factor (CF) | 1.0 | 1.1 | 1.1 | 1.0 | 1.1 | 1.6 |
| Hepatosomatic index (HSI) | 0.03 | 0.06 | 0.02 | 0.03 | 0.02 | 0.02 |

Tadpoles were collected in winter (in June, 2016 and September, 2017) and in winter (in January 2017). After summer tadpoles were habituated in laboratory conditions to 22 °C for 7 days, they were acclimated to 22 °C and 4 °C for 3 and 30 days. Developmental stages of the tadpoles in each group (n = 8) were recorded according to the criteria of Taylor and Kollros (1946). Condition factor was estimated using following equation. $CF = 100 * (\text{Body weight})/(\text{Body length})^3$. Hepatosomatic index was calculated by following equation. HSI = (Liver weight)/(Body weight). Data were expressed as means ± SEM. Different letters denote significant different means between the winter and summer samples or between 22 °C and 4 °C ($p < 0.05$).

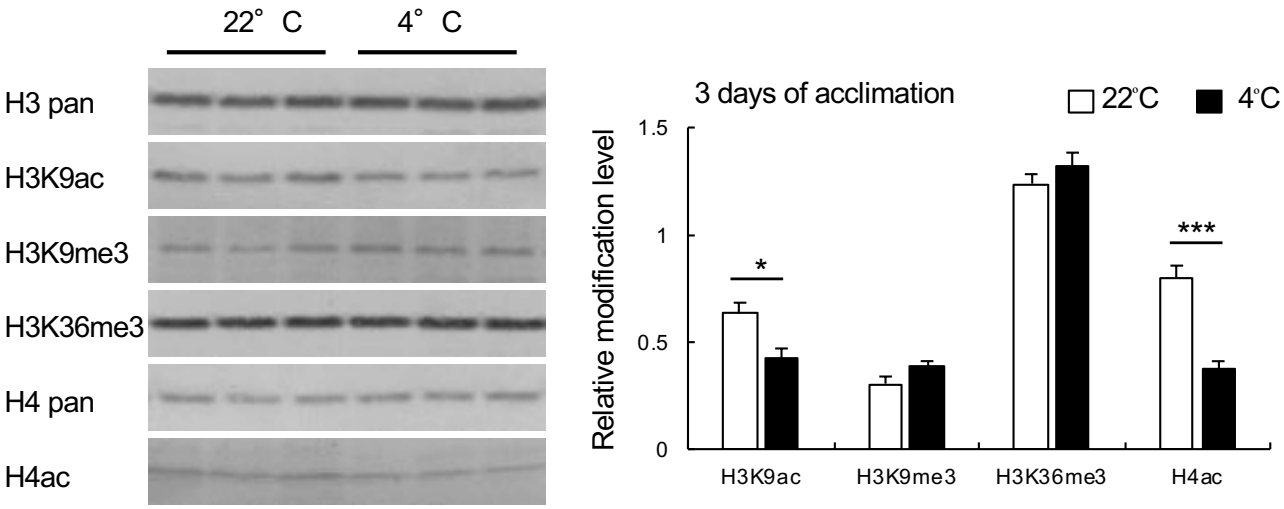
Table 2. Contents of free coenzyme A (CoA-SH) and acetyl coenzyme A (acetyl-CoA) in the bullfrog tadpole liver.

| Biological sample | CoA-SH | Acetyl-CoA | Acetyl-CoA/CoA-SH |
|---|---|---|---|
| Seasonal acclimatization | | | |
| Summer (22–30 °C) | 7.21 ± 0.88 (3) | 6.63 ± 0.39[a] (3) | 0.93 ± 0.06[a] (3) |
| Winter (5–14 °C) | 7.46 ± 0.72 (3) | 9.82 ± 0.34[b] (3) | 1.32 ± 0.09[b] (3) |
| Thermal acclimation (3 days) | | | |
| 22 °C | 12.55 ± 0.78 (4) | 10.18 ± 1.24[a] (4) | 0.81 ± 0.08[a] (4) |
| 4 °C | 12.80 ± 1.00 (5) | 17.06 ± 1.96[b] (4) | 1.38 ± 0.04[b] (4) |

Data were expressed as means ± SEM (n = 3–5) (nmol/g wet weight). Different letters denote significantly different means between two groups (summer vs. winter, and 22 °C vs. 4 °C) ($p < 0.05$) .

Fig. 1

A


22° C
4° C
H3 pan
H3K9ac
H3K9me3
H3K36me3
H4 pan
H4ac
3 days of acclimation
22°C
4°C
Relative modification level
1.5
1
0.5
0
*
***
H3K9ac
H3K9me3
H3K36me3
H4ac

B

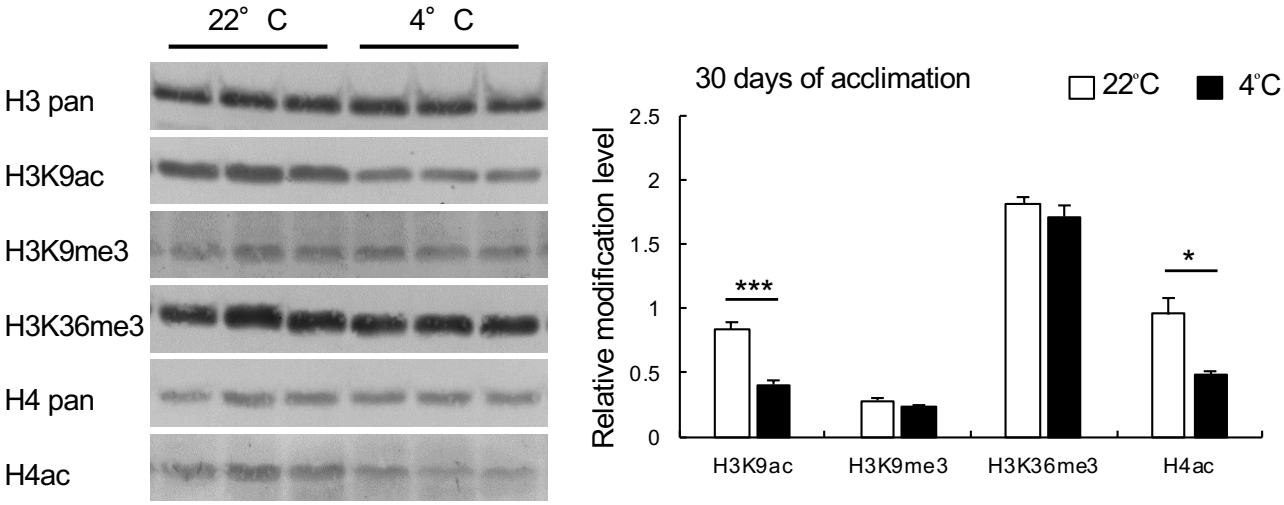

22° C
4° C
H3 pan
H3K9ac
H3K9me3
H3K36me3
H4 pan
H4ac
30 days of acclimation
22°C
4°C
Relative modification level
2.5
2
1.5
1
0.5
0
***
*
H3K9ac
H3K9me3
H3K36me3
H4ac

Fig. 2

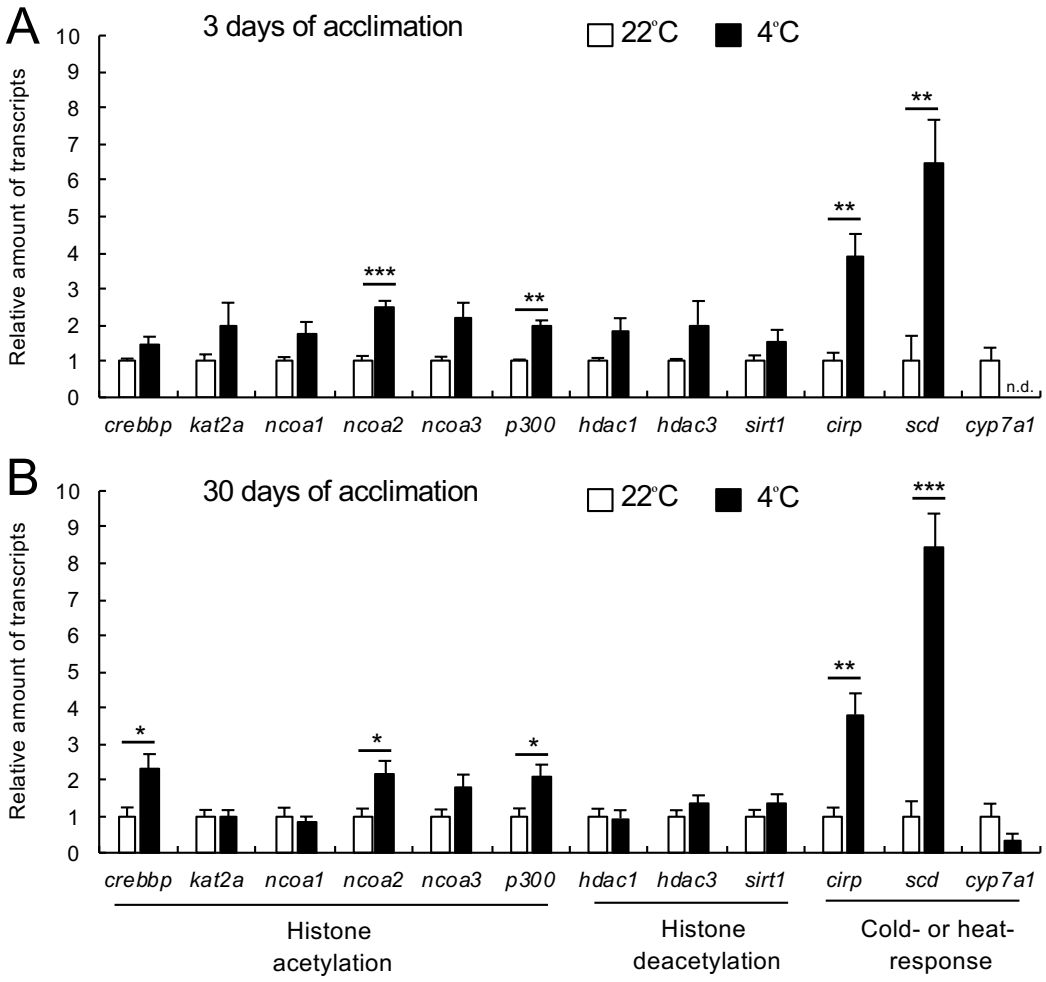
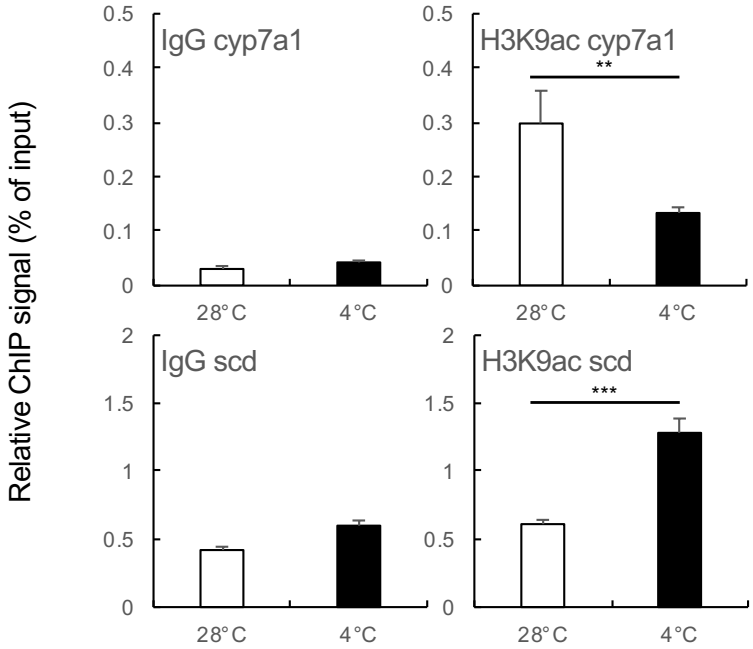

A
3 days of acclimation
22°C
4°C
Relative amount of transcripts
10
9
8
7
6
5
4
3
2
1
0
***
**
**
**
n.d.
crebbp
kat2a
ncoa1
ncoa2
ncoa3
p300
hdac1
hdac3
sirt1
cirp
scd
cyp7a1
B
30 days of acclimation
22°C
4°C
Relative amount of transcripts
*
*
*
**
***
crebbp
kat2a
ncoa1
ncoa2
ncoa3
p300
hdac1
hdac3
sirt1
cirp
scd
cyp7a1
Histone acetylation
Histone deacetylation
Cold- or heat-response

Fig. 3


Relative ChIP signal (% of input)
IgG cyp7a1
H3K9ac cyp7a1
**
IgG scd
H3K9ac scd
***
0.5
0.4
0.3
0.2
0.1
0
2
1.5
1
0.5
0
28°C
4°C