A Unique Energy-Saving Strategy during Hibernation Revealed by Multi-Omics Analysis in the Chinese Alligator

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HIGHLIGHTS
Metabolic and physiological pathways are overall suppressed during hibernation

Suppressed fat catabolism with active CPT1A suggests a unique energy-saving strategy

Hibernation-related genes are controlled by methylation-dependent transcription network

miRNAs play complex post-transcriptional regulation roles during hibernation

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SUMMARY

Many ectotherms hibernate in face of the harsh winter conditions to improve their survival rate. However, the molecular mechanism underlying this process remains unclear. Here, we explored the hibernation mechanism of Chinese alligator using integrative multi-omics analysis. We revealed that (1) the thyroid hormone biosynthesis, nutrition absorption and metabolism, muscle contraction, urinary excretion and immunity function pathways are overall downregulated during hibernation; (2) the fat catabolism is completely suppressed, contrasting with the upregulation of hepatic fatty-acid-transporter CPT1A, suggesting a unique energy-saving strategy that differs from that in hibernating mammals; (3) the hibernation-related genes are not only directly regulated by DNA methylation but also controlled by methylation-dependent transcription networks. In addition, we identified and compared tissue-specific, species-specific, and conserved season-biased miRNAs, demonstrating complex post-transcriptional regulation during hibernation. Our study revealed the genetic and epigenetic mechanisms underlying hibernation in the Chinese alligator and provided molecular insights into the evolution of hibernation regulation.

INTRODUCTION

Many reptiles and amphibians survive winter in refuges where they enter a state of dormancy, which allows them to substantially save energy. This is often referred to as hibernation, although the process itself is very different from that in mammals (Staples, 2016), as the body temperature of ectotherms is cooled to an ambient temperature by the Q10 effect, rather than through blocking thermoregulatory heat production (Grigg and Kirshner, 2015). Nonetheless, akin to mammals, the metabolic rates of hibernating ectotherms are strongly suppressed and their physical states and cardiovascular functions are dramatically altered (Herbert and Jackson, 1985; Storey, 1996) to a level that exceeds the passive effects of cooling, processes that are critical for their long-term survival during hibernation.

The Chinese alligator (Alligator sinensis) is a critically endangered freshwater crocodilian endemic to China (Wan et al., 2013) that diverged from the American alligator (Alligator mississippiensis) 31–58 million years ago (Oaks, 2011). Both species of the Alligator genus live at higher, thus cooler, latitudes than other crocodylian species and enter hibernation so as to survive the cold winter (Chen et al., 2003; Grigg and Kirshner, 2015). Typically, the Chinese alligator stops eating and goes into hibernation when temperatures drop in late October until late March (Chen et al., 2003; Fang et al., 2015). During this time, their metabolism is strongly suppressed and the animals sleep continuously unless they are disturbed (Fang et al., 2015). The quality of this hibernation period (as defined by undisturbed sleep in a temperature-appropriate environment) is known to exert a crucial impact on their health (Chen et al., 2003; Xia et al., 2006; Zhang et al., 2003). In addition to human hunting and habitat disruption, global climate change is becoming a critical threat to Chinese alligators, as indeed it is for many other hibernators (Humphries et al., 2002; Inouye et al., 2003). Therefore, exploring the gene regulatory network underlying seasonal physiological changes is not only important for revealing how hibernating ectotherms overcome the cold and foodless winter in their habitat but may also ultimately aid their conservation in the future.

Various studies over the past half century have investigated the molecular mechanisms underlying hibernation, and numerous associated genes and pathways have been identified (Storey, 2006; Storey and...
Epigenetic mechanisms mediate gene-environment interactions in various biological processes. DNA methylation is an ancient epigenetic modification in eukaryotic genomes that plays essential roles in various biological processes, including the regulation of gene expression, development, and stress responses (Breiling and Lyko, 2015; Pelizzola and Ecker, 2011; Smith and Meissner, 2013; Su et al., 2011). The level and pattern of DNA methylation typically varies among species and cell types. Usually, promoter methylation is negatively correlated with gene transcription levels, whereas methylation in the gene body is associated with active transcription (Jones, 2012). DNA methylation reportedly plays an important role in the regulation of gene expression associated with mammal hibernation (Alvarado et al., 2015; Biggar and Storey, 2014b; Fujii et al., 2006); however, previous studies largely focused on changes in overall DNA methylation levels, or methylation of specific genes. MicroRNAs (miRNAs) are another epigenetic mechanism involved in hibernation (Arfat et al., 2018; Biggar and Storey, 2015; Lyons et al., 2013). DNA methylation is associated with gene transcription potential, whereas miRNAs participate in post-transcriptional regulation. Although animal miRNAs are phylogenetically conserved (Ambros, 2004), seasonal expression changes vary among species and even tissues (Arfat et al., 2018; Biggar and Storey, 2015; Lyons et al., 2013).

We collected tissues and serum samples from adult Chinese alligators in winter (the coldest time of the year in the Chinese alligator habitat) and summer (the active season of the Chinese alligator) and analyzed them using mRNA-seq, bisulfite sequencing (BS-seq), small RNA sequencing (sRNA-seq), and iTRAQ/TMT protein analysis to comprehensively explore the genetic and epigenetic mechanisms underlying reptile hibernation (Figure 1, Table S1, Supplementary Text).

RESULTS

Energy Metabolism Is Suppressed during Hibernation

To gain insights into the molecular mechanisms that enable the Chinese alligator to substantially save energy during hibernation, we compared the transcriptomes of tissues and proteomes of serum collected...
during winter and summer periods. The seasonal transcriptome variations were matched to the biological functions of different tissues. In order to correlate this with the BS-seq and sRNA-seq data, we focused on the transcriptomes of the female alligator in most of the subsequent analyses. However, we note that gene expression patterns were largely similar in tissues from male and female animals.

**Thyroid Hormone Biosynthesis and Signaling**

The hypothalamus-pituitary-thyroid axis is essential for metabolism regulation. Our results revealed that both the thyrotropin-releasing hormone gene (TRH) in the hypothalamus and the thyroid hormone biosynthesis pathway in the thyroid glands are downregulated during hibernation. KEGG pathway analysis revealed that the “thyroid hormone biosynthesis pathway” was enriched in downregulated differentially expressed genes (DEGs) during hibernation (winter-suppressed DEGs) in the thyroid gland (q < 0.05) (Table S2). Various genes involved in thyroid hormone biosynthesis, including TSHR, TG, and TPO, are suppressed in winter (Figure 2A). Correspondingly, serum levels of thyroid hormones (T3, T4, free T3, and free T4), TG antibody, and TPO antibody are substantially lower in winter (Table S3). iTRAQ/TMT analysis of serum samples indicated that von Willebrand factor (VWF), cartilage oligomeric matrix protein (COMP), and actin 5 (ACT5) in the “thyroid hormone signaling pathway” are significantly downregulated in winter (Table S4). We assumed that fuel use and physiological states would be altered significantly during hibernation (see below) owing to the downregulation of the thyroid hormone.

**Digestion and Absorption**

Food digestion and absorption largely take place in the small intestine, and fat is digested with the aid of bile acid synthesized by the liver. KEGG pathway analysis revealed that “primary bile acid biosynthesis” (liver), “fat digestion and absorption,” “vitamin digestion and absorption,” and “mineral absorption” (small intestine) were significantly enriched (q < 0.05) in winter-suppressed DEGs (Figures 2B and S1A, Table S2). Significantly more downregulated than upregulated DEGs (Wilcoxon signed-rank test, q < 0.05) were observed in digestion- and absorption-related pathways (Figure S1). These results suggested that digestion and absorption in the small intestine and bile acid biosynthesis and secretion in the liver are suppressed during hibernation, which is consistent with the alligator’s fasting state in winter.

**Nutrient Metabolism**

Carbohydrates are the primary energy source in animals and are degraded for ATP production through glycolysis/glucoseogenesis, the citrate cycle (tricarboxylic acid cycle), and oxidative phosphorylation. Significantly more winter-suppressed than winter-activated DEGs (Wilcoxon signed-rank test, q < 0.05) were observed in these three pathways (Figures 2C and S2A). Particularly, genes encoding rate-limiting enzymes of glycolysis (6-PFK) and the citrate cycle (CS, IDH, and OGDH) were significantly downregulated during hibernation in most tissues (Figure 2C). Pyruvate kinase genes (KPM and KPLR) showed limited seasonal differences in most tissues, whereas KPM and KPLR proteins in the serum were significantly downregulated during hibernation (Table S3), suggesting post-transcriptional regulation of these two factors. Glycogen serves as a form of energy storage mainly in the liver and muscles. Glycogen serves as a form of energy storage, mainly in the liver and muscles. The expression of the liver glycogen synthase gene GYS1, but not that of the muscle glycogen synthase gene GYSM or glycogen phosphorylase genes (GYPL, GYPM, and GYPB), was significantly downregulated during hibernation (Figure 2C). Conversely, the expression of the muscle glycogen phosphorylase gene GYPM was considerably upregulated, although not statistically significantly (q = 6.85 × 10^{-36}, fold change = 1.48). These results suggested
During hibernation, the heart beats slower. Cardiac muscle contraction is a complex process initiated by $Ca^{2+}$ influx. Seven voltage-dependent calcium channel genes (CACNA1C-1, CACNA1C-2, CACNA2D2-1, CACNA2D2-2, CACNA2D1, CACNB4, and CACNG2) were downregulated in the hibernating alligator (Figures 2D and S3A). Interestingly, six pathogenicity genes involved in heart failure, i.e., RYR2, DSP, and PKP2 for arrhythmogenic right-ventricular cardiomyopathy, DMD and MYBPC3 for dilated cardiomyopathy, and MYH7 for hypertrophic cardiomyopathy, were suppressed during hibernation (Figures 2D and S3A). The downregulation of genes involved in cardiac muscle contraction provides a molecular mechanism underlying the alligator’s low heart rate during hibernation.

We also identified various season-biased DEGs involved in skeletal muscle function and development. PHKG2, MYLK3, KLHL40, and MYF6, involved in skeletal muscle development, were downregulated during hibernation (Figures 2E and S3B). TNNI1, TNNC1, TPM2, TPM3, MYL3, and MYH7, encoding slow-twitch skeletal muscle components, were also suppressed, whereas TNNC2, MYL1, and MYH3, encoding fast-twitch skeletal muscle components, were upregulated during hibernation (Figures 2E and S3B). These gene expression patterns are consistent with our previous finding that alligators show self-defense behavior when they are disturbed and awakened from hibernation but soon calm down and fall asleep again (Fang et al., 2015).

Urinary Excretion

With the significant decrease in metabolic gene expression, renal function-related gene expression was also expected to be suppressed during hibernation. Indeed, the KEGG pathways “collecting duct acid secretion” and “proximal tubule bicarbonate reclamation” were significantly enriched ($q < 0.05$) in
winter-suppressed DEGs in the kidneys (Table S2). These DEGs included genes encoding CA2, which catalyzes the reversible hydration of carbon dioxide; SNAT3, GLS, and GLUD, which transport and catalyze glutamine to produce NH₄⁺; and many transmembrane transporters in proximal tubular cells (NBC1, ATP1, ATP1B, and AQP1) and collecting duct intercalated cells (AE1, KCC4, CLCNKB, and seven ATPases), which transport ions across urine, cytoplasm, and blood. Various genes involved in the reabsorption of water, calcium, and sodium were downregulated, including AQP2 and ENAC, ENACB and ENACG, as well as VDR, PTHR, and KL, the protein products of which regulate Ca²⁺ channel expression and apical abundance (Figures 2F and S3C). These results indicated that kidney function is suppressed in the hibernating alligator.

Immunity

The spleen is the largest immune organ and is a reservoir of macrophages and lymphocytes, which act as scavengers and in immune defense against pathogens, respectively. Winter-suppressed DEGs in the spleen were enriched in KEGG pathways involved in immunity and hematopoiesis (Table S2). In particular, the downregulated DEGs included genes crucial for lymphocyte production, including ADD, IL7R (NK-cell and pro-T-cell production), CD3E, CD45, CD8A (T cell production), IGH, BTK, and TACI (B-cell production) (Figure 2G), suggesting that lymphocyte proliferation is suppressed during hibernation. Various genes encoding lysosomal acid hydrolases and lysosomal membrane proteins were significantly downregulated. Genes encoding phagosomal components, including MPO, NCF2, NCF4, and CTL, were downregulated, indicating that phagocytosis is suppressed during hibernation (Figure 2G). In addition, various winter-suppressed DEGs are involved in infectious diseases (leishmaniasis, malaria, asthma, tuberculosis, pathogenic Escherichia coli infection, etc.) (Figure 2G, Table S2). These results suggested that, as the hibernating Chinese alligator stays in a refuge, where less exogenous pathogens are present, immunity and hemopoiesis are suppressed to save energy.

Factors Actively Upregulated during Hibernation

Several factors actively upregulated during hibernation were identified in the Chinese alligator. The expression of cold-inducible RNA-binding protein genes (CIRBP) can be induced simply by cold stress (Saito et al., 2000; Sugimoto and Jiang, 2008). In nearly all tissues, the transcription of CIRBP was actively upregulated, especially in the brain, thyroid, liver, small intestine, adipose tissues, kidneys, heart, and skeletal muscle (Figure S3D). These results suggested that CIRBP plays a critical, although not fully elucidated, role in the ectotherm hibernator. Unexpectedly, c-FOS, a neural activity marker gene (Mateju et al., 2009), was also overexpressed during hibernation in most central and peripheral organs, except the thyroid gland (Figure S3E), suggesting active neural states in the hibernating alligator and its positive regulation during hibernation. Furthermore, general transcription factor (GTF) genes were generally activated during winter (Wilcoxon signed-rank test, \( q = 7.896\times10^{-5} \)) (Figure S3F). Thus, the downregulation of genes participating in thyroid hormone biosynthesis, nutrient absorption and metabolism, urinary excretion, and immunity during hibernation is likely not simply regulated via GTF gene repression but by more specific and complex pathways, such as DNA methylation and miRNAs.

DNA Methylation Landscapes in the Hibernating Chinese Alligator

An average of 24.43 M methylated cytosines (mCs) were identified by BS-seq in each tissue sample, accounting for 2.49% of cytosines (Cs) in the Chinese alligator. Most mCs (96.66%) were in CpG context (Figure 3A), whereas mCs in CHG and CHH contexts were rare and were substantially less methylated (Figures 3A and 3B), consistent with findings in other vertebrates (Figure 3C). Therefore, we focused on CpG sites in most of the subsequent analyses.

DNA methylation patterns varied among genome regions. In transcribed regions and ~2 kb upstream and downstream of these regions, CG methylation levels were lowest in the promoter, where the methylation level gradually declined to a minimum at the transcription start site (TSS) and then increased again in the 5’ UTR. CG methylation levels were highest in the exon and intron regions but slightly decreased in the 3’ UTR (Figure 3D). These results suggested that DNA methylation may participate in the regulation of transcription initiation. We also evaluated methylation levels in GC islands, microsatellites, transposable elements, and their adjacent regions. The relatively higher CG methylation levels within transposable elements suggested suppression of active transposons (Figure 3E). No significant difference was found in global DNA methylation patterns between winter and summer samples in terms of methylation broadness and
deepness levels ($p > 0.05$), suggesting that the regulation of DNA methylation in hibernation is not simply through overall hyper- or hypomethylation (Figure 3F, Figure S4).

**Correlation between DNA Methylation Status and Gene Expression**

To explore potential regulatory roles of DNA methylation in gene expression in the hibernating Chinese alligator, we correlated mRNA-seq data with BS-seq data obtained from the same tissues. Promoter CG methylation was negatively correlated with gene expression. As for CG methylation in the gene body and downstream thereof, non-expressed genes had the lowest DNA methylation levels, whereas genes with intermediate expression demonstrated the highest DNA methylation levels (Figure 4A). Genes with the highest gene-body methylation levels (fourth and fifth groups in Figure 4B) tended to be expressed at intermediate levels. These results suggested that promoter hypermethylation is associated with transcriptional repression, whereas gene-body methylation plays a role in the normalization of gene overexpression.

To explore DNA methylome alterations in the hibernating Chinese alligator, we identified differentially methylated genes (DMGs) between winter and summer samples. DEGs were significantly enriched among
Figure 4. DNA Methylation Regulation of Gene Expression during Chinese Alligator Hibernation

(A) Distributions of methylation levels within gene bodies and 2-kb upstream and downstream regions by different expression levels. Based on the expression level, protein-coding genes were divided into two groups: non-expressed genes (FPKM < 1) and expressed genes. The latter were further divided into three groups: low-expression genes (FPKM < lower quartile), intermediate-expression genes (upper quartile < FPKM < upper quartile), and high-expression genes (FPKM > upper quartile).

(B) Expression profiles of methylated and unmethylated genes. Methylated genes were further divided into five groups based on the methylation level in their gene body (20% quintiles).
DMGs in most tissues (q < 0.05), except the kidneys (q = 0.054) (Figure 4C), suggesting that DNA methylation does regulate transcription. Some genes involved in physiological function regulation in hibernation exhibited altered methylation. For example, CYP39A transcription was significantly suppressed in the liver during hibernation, with a hyper-differentially methylated region (DMR) from promoter to gene body (Figure 4D). Both HLHL40 and NCOR2 were hypermethylated in the gene body, but HLHL40 was downregulated in hibernating skeletal muscle, whereas NCOR2 was upregulated in the liver (Figure 4D). These results indicated that DNA methylation is, at least in part, responsible for adaptive transcriptional changes during hibernation, but its roles are much more complicated than previously realized. Indeed, the correlations between hyper- and hypo-DMGs, and down- and upregulated DEGs, were substantially more complex than anticipated. For example, 45 genes were hypermethylated and downregulated, whereas 31 genes were hypomethylated and upregulated in the liver during hibernation. However, 31 hyper-DMGs and 46 hypo-DMGs were up- and downregulated, respectively (Figure 4E).

Furthermore, a large number of DEGs (2,197) did not overlap with DMGs (Figure 4E), suggesting that seasonal changes in the expression of these genes may not be directly regulated by DNA methylation but rather result from methylation-dependent alterations in transcription networks.

**DNA Methylation Alterations in TFs and Their Regulatory Networks during Hibernation**

To investigate the role of methylation alterations in gene regulatory networks during hibernation, we subjected the season-biased DMGs to GO analysis, which revealed that, in nearly all tissues, GO terms referring to regulation of gene expression and metabolic process were significantly enriched (q < 0.05) in season-biased DMGs (Figure 5A). We identified 1,370 transcription factor (TF) genes in the Chinese alligator genome and analyzed them in relation to seasonal DMGs in each tissue. The observed ratio of differentially methylated TFs versus DMGs was significantly higher than expected (1,370/27,500) (Fisher’s exact test, q < 0.05) (Figure 5B), suggesting that genes involved in gene expression regulation, especially TF genes, are more likely to be differentially methylated. To explore methylation-dependent regulatory networks in the hibernating Chinese alligator, we identified differentially expressed TFs with DMRs in each tissue (Figure 5C). Furthermore, we performed weighted gene co-expression network analysis (WGCNA) based on transcriptome data and constructed methylation-dependent regulatory networks for each tissue (Figures 5D, SE, and SS). Most of the season-biased DEGs were regulated by the differentially methylated TFs in the transcription networks.

**miRNA Regulation during Hibernation**

miRNAs reportedly play a crucial role in gene expression regulation during hibernation (Arfat et al., 2018; Biggar and Storey, 2015; Lyons et al., 2013). Thus, we carried out sRNA-seq using adipose, brain, heart, small intestine, muscle, and gonad tissues to identify season-biased differentially expressed miRNAs (DEmiRs, q < 0.05). Some hibernation-related miRNAs are conserved and play roles in other hibernators. For example, miR-103, miR-124 (brain), and miR-206 (skeletal muscle) were upregulated in the hibernating alligator (Figure 6A), and these expression changes were also reported in corresponding tissues of the hibernating little brown bat (Myotis lucifugus) (Biggar and Storey, 2014a; Kornfeld et al., 2012). We also discovered new hibernation-related miRNAs, some of which are specific to the Chinese alligator (Figure 6A). The roles of miRNAs in gene expression regulation were found to be tissue specific. Their expression levels varied in different tissues during hibernation (Figures 6B–6D). For example, miR-10b was upregulated in the small intestine and adipose tissues during hibernation but downregulated in the brain, heart, muscle, and ovaries (Figure 6C). Similar patterns were observed for miR-1a and miR-19a (Figures 6B and 6D). Furthermore, the expression patterns suggested that the roles of some miRNAs are species specific. MiR-200a was downregulated in the adipose tissues of the hibernating Chinese alligator (Figure 6A) but reportedly is upregulated in hibernating thirteen-lined ground squirrels (Ictidomys tridecemlineatus) (Wu et al., 2014). MiR-1a is upregulated in the muscle in several hibernators (Arfat et al., 2018; Biggar and Storey, 2015) but not in the Chinese alligator.
To explore the regulatory roles of annotated miRNAs during hibernation in the Chinese alligator, we predicted target genes of season-biased DEmiRs. Various genes involved in functional regulation during hibernation in each tissue were targeted by miRNAs (Table S5), including DGAT1, DGAT2, APOA1, APOA4, APOB, ABCG8, CD36, MTP1, MTP2, FABP2 and SCARB1, which are related to fat digestion and absorption and were downregulated in the small intestine (Figure 6E), and LPL, FATP6, SCD, and GOT2, which are related to lipid metabolism and were downregulated in adipose tissues during hibernation (Figure 6F). One gene could be regulated by multiple miRNAs and, conversely, one miRNA could target multiple genes, thus forming a complex regulatory network (Figures 6E and 6F).

**DISCUSSION**

Although hibernation in ectotherms seems quite similar to that in endotherms, they are quite different in behavioral, physiological, and biochemical traits (Grigg and Kirshner, 2015; Staples, 2016). In hibernating mammals, the metabolic rate is suppressed during winter, along with a decrease in the core body temperature, which results in substantial energy saving (Hampton et al., 2013; Staples, 2016); however, periodic interbout arousals during hibernation still consume much energy (Karpovich et al., 2009). On the other hand, ectotherm hibernators exhibit continuous metabolic suppression exceeding the passive thermal effects due to a decrease in the environmental temperature in winter (Grigg and Kirshner, 2015; Staples, 2016).

![Figure 5](image_url)
Figure 6. MiRNA Regulation of Gene Expression during Chinese Alligator Hibernation

(A) Functional key genes targeted by season-biased differentially expressed miRNAs (DEmiRs) in each tissue.

(B–D) Seasonal expression changes for miR-1a (B), miR-10b (C), and miR-19a (D).

(E and F) Relations between DEmiRs and differentially expressed genes (DEGs) in fat digestion and absorption in the small intestine (E) and lipid metabolic pathway genes in adipose tissues (F).

See also Table S5.
In mammals, the entering of hibernation is accompanied by a switch of fuel use from carbohydrates to lipids, which provide the most energy-dense metabolic substrate (Sheriff et al., 2013). Previous transcriptome studies in hibernating mammals have provided molecular evidences for this switch. For example, in the white adipose tissue of hibernating free-ranging dwarf lemurs in a wild population, several genes involved in lipid catabolism (e.g., PLPP1, APOC2, SCD, FASN, and ELOVL6) and carbohydrate oxidation repression (PKDH4) were induced, whereas several genes involved in carbohydrate catabolism (e.g., PDHA1, PDHB, DLAT, and DLD) were downregulated (Faherty et al., 2018). In the brown adipose tissue of hibernating thirteen-lined ground squirrel, many genes participating in lipolysis (e.g., PNP/L2A, PLIN2, PLIN4, and PLIN5) and lipid transport (e.g., OBP2B, FABP3, SLC25A20, CPT1A, and CPT2), as well as PDK4 are upregulated (Hampton et al., 2013). In addition, in the past three decades, many studies on gene expression, epigenetic regulation, proteins, enzymes, and posttranslational modification have provided evidence supporting the switch of fuel use in mammalian hibernators (Carey et al., 2003; Staples, 2016).

The main energy source and the energy-saving strategy of hibernating ectotherms are still unclear. Similar to mammal hibernators, the common lizard (Lacerta vivipara) and gecko (Phyllodactylus marmoratus) reportedly rely on fat stores during hibernation (Avery, 1970; Daniels, 1984), whereas another study suggested that glycogen, not lipids, limits winter survival of side-blotched lizards (Uta stansburiana) (Zani et al., 2012). Both liver- and muscle-stored glycogens are substantially consumed during hibernation and considerably account for the winter energy budget of several lizard and snake species (Costanzo, 1985; Dessauer, 1953; Zani et al., 2012). Transcriptomic studies on the heart, skeletal muscle, and kidneys/brain of Chinese alligator and Australian central bearded dragon have identified candidate genes and pathways that are involved in seasonal adaption and tissue-specific function maintenance and have provided valuable insights into the molecular regulatory mechanism underlaying reptile hibernation (Capraro et al., 2019; Sun et al., 2018). In our study, we extend the analysis to other important tissues, including the brain, hypothalamus (central control), thyroid gland (metabolism regulation), small intestine (nutrient digestion and absorption), liver (metabolism), adipose (energy storage), lung (gas exchange), heart (blood supply), skeletal muscle (movement), and spleen (immunity), to provide a comprehensive transcriptome profile of the hibernating reptile.

Our data revealed a unique energy-saving strategy in the hibernating Chinese alligator during hibernation. Adapting to the fasting state, the hibernating alligator suppressed pathways related to nutrition absorption and metabolism. Overall, genes in fat catabolism pathways were dramatically downregulated in the hibernating Chinese alligator, except for liver CPT1A, which was significantly upregulated, suggesting that the fat metabolism pathways are generally suppressed instead of activated during alligator hibernation. However, β-oxidation of FA in the liver, but not in the muscle, was activated to use the limited FAs, ensuring the energy demands of the liver as the metabolic center. This is supported by our finding during tissue collection that the amount of adipose tissue did not differ significantly between winter and summer. In addition, glycogen phosphorylase genes were not downregulated like other genes in carbohydrate metabolism; instead, the muscle glycogen phosphorylase gene GYPM was considerably upregulated (q = 6.85 × 10^-36, fold change = 1.48), suggesting that glycogen, especially that stored in muscle, may be another reserve energy source of the hibernating Chinese alligator. Our results provide molecular evidence that glycogenolysis in muscle and β-oxidation of hepatic FA supply scarce energy for the hibernating Chinese alligator with suppressed carbohydrate and fat catabolism. Through downregulation of thyroid-releasing hormone gene and its downstream thyroid hormone biosynthesis pathway, nutrition absorption and metabolism, cardiac and show skeletal muscle contraction, and urinary excretion and immunity function pathways were also generally downregulated during hibernation, reflecting a coordinated suppression of the metabolic rate and physiological states. However, a few upregulated genes in these pathways, for example, CPT1A and AMPK, which, respectively, catalyze FA catabolism and inhibit FA synthesis in the liver, as well as upregulated genes involved in fast muscle fiber contraction (TNNC2, MYL1, and MYH3), reveal the ingenious energy utilization and survival strategies in this species.

In Australian central bearded dragon, the enrichment of “lipid catabolic processes” and “carbohydrate catabolic processes” GO terms in downregulated genes suggest an overall suppression of lipid and carbohydrate catabolism, but the upregulation of carbohydrate metabolism genes, such as PFKFB3, GSK3A, and FBP1, which are important in glycolysis, glycogen synthesis, and gluconeogenesis, respectively, indicates a different strategy of fuel use and energy saving (Capraro et al., 2019). Using seasonal transcriptome data from the kidneys, skeletal muscle, and heart in the Chinese alligator reported by another research group (Sun et al., 2018), we were able to explore whether the adaptive mechanisms are similar between the only two Chinese alligator populations. We identified five DEGs (62.5%) among the eight seasonal DEGs reported in their study, including CSRP3, AT1A1,
PCKGC, KCRB, and CIRBP. In addition, the two datasets share many of the KEGG pathways enriched in seasonal DEGs. These results support the repeatability and universality of our data.

DNA methylation reportedly plays an important role in the regulation of gene expression associated with mammalian hibernation (Alvarado et al., 2015; Biggar and Storey, 2014b; Fuji et al., 2006). TFs read DNA methylation and translate the information into certain gene expression patterns (Buck-Koehntop and Defossez, 2013; Zhu et al., 2016). TF genes themselves are also DNA methylation targets in various biological processes (Ivascu et al., 2007; Zinger et al., 2019). In thirteen-lined ground squirrels, the global DNA methylation level was increased in brown adipose tissue (Biggar and Storey, 2014b) but decreased in skeletal muscle (Alvarado et al., 2015). The CpG methylation level of the MEF2C promoter region correlated with the downregulation of gene expression in skeletal muscle of thirteen-lined ground squirrels (Alvarado et al., 2015). In chipmunk, CpG methylation in the USF-binding site is crucial for liver-specific transcription of the hibernation-specific gene, HP-27 (Fuji et al., 2006). Although these studies provided a glance into the DNA regulation in hibernation, they largely focused on changes in overall DNA methylation levels or the methylation of specific genes. In this study, we carried out BS-seq, the gold standard for DNA methylation profiling with high resolution, in active and hibernating states. By combining transcriptome and methylome data, we found that cis- and trans-regulation of DNA methylation participates in gene transcription changes during hibernation. Although DMGs are likely to be differentially expressed, most DEGs are not directly regulated by DNA methylation changes but by differentially expressed TFs with DMRs. In this economical and ingenious strategy, reversible drastic transcriptome changes in the Chinese alligator are regulated by modulating the transcription of TF genes. These results are consistent with observations in other hibernators, where numerous TFs play important roles during hibernation, such as HNF-1 in the chipmunk (Tamias asiaticus) (Ono et al., 2001), ATF4 and NFAT in the thirteen-lined ground squirrel (Mamady and Storey, 2008; Zhang and Storey, 2016), ZBED1 in the greater horseshoe bat (Rhinolophus ferrumequinum) (Xiao et al., 2016), and HSF in the red-eared slider turtle (Trachemys scripta elegans) (Kvorturzho and Storey, 2010).

Overall, our results provide insights into the genetic and epigenetic mechanisms underlying hibernation in the Chinese alligator and are expected to facilitate the development of scientific programs for successful conservation of this endangered species.

Limitations of the Study
This study revealed the suppression of metabolic rate and physiological states of hibernating Chinese alligator and suggested a unique energy-saving strategy that differs from that in hibernating mammals. Although we found that the downregulation of thyroid hormone biosynthesis plays an important regulatory role during this process, more investigations are needed to identify the core “hibernation factor,” which triggers and turns off the hibernation state. In addition, similar omics study should be done on more hibernating ectotherms to further explore the evolution of hibernation regulation in hibernating animals including reptiles as well as other ectotherms.

Resource Availability
Lead Contact
Further information and requests for resources should be directed to and will be fulfilled by the Lead Contact, Sheng-Guo Fang (sgfanglab@zju.edu.cn).

Materials Availability
The study did not generate new unique reagents.

Data and Code Availability
The Chinese alligator reference genome is available in the NCBI with the assembly accession number GCA_000455745.1. The BS-seq, mRNA-seq, and sRNA-seq data generated in this work have been deposited in the SRA database under NCBI BioProjects: PRJNA556094, PRJNA556093, and PRJNA556092, respectively. The iTRAQ and TMT data have been deposited in the ProteomeXchange with identifier PXD019278 and PXD019277, respectively. The DNA methylome data of Petromyzon marinus, Danio rerio, Bos grunniens, Homo sapiens, and Gallus gallus were downloaded from NCBI SRA database: SRR2457525, SRR800080, SRR8834688, SRR3427332, and SRR5003428, respectively.
METHODS
All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION
Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2020.101202.

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AUTHOR CONTRIBUTIONS
Conceptualization, S.-G.F. and Q.-H.W.; Methodology, Q.-H.W. and J.-Q.L; Investigation and Formal Analysis, J.-Q.L., Y.-Y.H., M.-Y.B., Q.-H.W., and S.-G.F.; Resources, S.-G.F.; Writing – Original Draft Preparation, J.-Q.L., M.-Y.B., Q.-H.W., and S.-G.F.; Writing – Review & Editing, J.-Q.L., Y.-Y.H., Q.-H.W., and S.-G.F.; Supervision, S.-G.F. and Q.-H.W.; Project Administration and Funding Acquisition, S.-G.F.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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Supplemental Information

A Unique Energy-Saving Strategy
during Hibernation Revealed by Multi-Omics

Analysis in the Chinese Alligator

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Figure S1. Gene expression patterns in key pathways involved in digestion and absorption in the liver and small intestine, Related to Figure 2. A, B. Season-biased differentially expressed genes (DEGs) in key pathways involved in nutrient digestion and absorption in the liver and small intestine in female (A) and male (B) Chinese alligators. C. Expression heatmap of seasonally DEGs in key pathways involved in digestion and absorption in the liver and small intestine.
Figure S2. Gene expression patterns in key pathways involved in nutrient metabolism,
Related to Figure 2. A. Box plots of season-biased differentially expressed gene (DEG) numbers in pathways involved in carbohydrate metabolism. B. Expression heatmap of season-biased DEGs involved in lipid metabolism in the liver, adipose tissues, cardiac muscle, and skeletal muscle. C. Numbers of DEGs in amino acid metabolic pathways in the liver. D. Expression patterns of genes crucial for amino acid metabolism.
Figure S3. Gene expression patterns in key pathways involved in physiological function,

Related to Figure 2. A, B, C. Heatmaps of differentially expressed genes (DEGs) involved in cardiac contraction and heart disease (A), skeletal muscle contraction and development (B), reabsorption and excretion in the kidneys (C). D, E. Expression patterns of CIRBP (D) and c-FOS (E). F. Comparison of the numbers of upregulated and downregulated general transcription factor genes (GTFs) during hibernation.
Figure S4. Comparison of DNA methylation in CG context in inactive (winter) and active (summer) periods, Related to Figure 3.
Figure S5. Associations between transcription factors (TFs) and key functional genes in each tissue, Related to Figure 5.
Table S1. Sample names for bisulfite sequencing, mRNA sequencing, and small RNA sequencing, Related to Figure 1.

|                | mRNA sequencing | Bisulfite sequencing | Small RNA sequencing |
|----------------|-----------------|----------------------|---------------------|
|                | Winter | Summer | Winter | Summer | Winter | Summer |
| Liver          | Male    | Female | Male    | Female | Male    | Female |
| Winter         | WM_LIV  | WF_LIV | SM_LIV  | SF_LIV | WF_LIV  | SF_LIV |
| Summer         | WM_LIV  | WF_LIV | SM_LIV  | SF_LIV | WF_LIV  | SF_LIV |
| Adipose        | Male    | Female | Male    | Female | Male    | Female |
| Winter         | WM_ADI  | WF_ADI | SM_ADI  | SF_ADI | WF_ADI  | SF_ADI |
| Summer         | WM_ADI  | WF_ADI | SM_ADI  | SF_ADI | WF_ADI  | SF_ADI |
| Heart          | Male    | Female | Male    | Female | Male    | Female |
| Winter         | WM_HEA  | WF_HEA | SM_HEA  | SF_HEA | WF_HEA  | SF_HEA |
| Summer         | WM_HEA  | WF_HEA | SM_HEA  | SF_HEA | WF_HEA  | SF_HEA |
| Brain          | Male    | Female | Male    | Female | Male    | Female |
| Winter         | WM_BRA  | WF_BRA | SM_BRA  | SF_BRA | WF_BRA  | SF_BRA |
| Summer         | WM_BRA  | WF_BRA | SM_BRA  | SF_BRA | WF_BRA  | SF_BRA |
| Hypothalamus   | Male    | Female | Male    | Female | Male    | Female |
| Winter         | WM_SPL  | WF_SPL | SM_SPL  | SF_SPL | WF_HYP  | SF_HYP |
| Summer         | WM_SPL  | WF_SPL | SM_SPL  | SF_SPL | WF_HYP  | SF_HYP |
| Spleen         | Male    | Female | Male    | Female | Male    | Female |
| Winter         | WF_HYP  | SF_HYP | WF_SPL  | SF_SPL | WF_HYP  | SF_HYP |
| Summer         | WF_HYP  | SF_HYP | WF_SPL  | SF_SPL | WF_HYP  | SF_HYP |
| Kidney         | Male    | Female | Male    | Female | Male    | Female |
| Winter         | WM_KID  | WF_KID | SM_KID  | SF_KID | WF_KID  | SF_KID |
| Summer         | WM_KID  | WF_KID | SM_KID  | SF_KID | WF_KID  | SF_KID |
| Lung           | Male    | Female | Male    | Female | Male    | Female |
| Winter         | WM_LUN  | WF_LUN | SM_LUN  | SF_LUN | WF_LUN  | SF_LUN |
| Summer         | WM_LUN  | WF_LUN | SM_LUN  | SF_LUN | WF_LUN  | SF_LUN |
| Skeletal muscle| Male    | Female | Male    | Female | Male    | Female |
| Winter         | WM_MUS  | WF_MUS | WM_MUS  | SF_MUS | WF_MUS  | SF_MUS |
| Summer         | WM_MUS  | WF_MUS | WM_MUS  | SF_MUS | WF_MUS  | SF_MUS |
| Small intestine| Male    | Female | Male    | Female | Male    | Female |
| Winter         | WM_INT  | WF_INT | SM_INT  | SF_INT | WF_INT  | SF_INT |
| Summer         | WM_INT  | WF_INT | SM_INT  | SF_INT | WF_INT  | SF_INT |
| Thyroid        | Male    | Female | Male    | Female | Male    | Female |
| Winter         | WF_THY1 / WF_THY2 | SF_THY1 / SF_THY2 | WF_THY1 | SF_THY1 |
Table S3. Serum thyroid hormone concentrations, related Figure 2.

|             | Active (summer) period | Inactive (winter) period | Unit   |
|-------------|------------------------|--------------------------|--------|
| T3          | >10.00                 | 0.95                     | nmol/L |
| T4          | 88.09                  | 6.42                     | nmol/L |
| FT3         | 18.83                  | 1.5                      | pmol/L |
| FT4         | 56.48                  | <0.30                    | pmol/L |
| TG-Ab       | 271.6                  | 20.04                    | KIU/L  |
| TPO-Ab      | >600.00                | 30.96                    | KIU/L  |
| TSH         | <0.005                 | <0.005                   | mIU/L  |
Table S4. Comparison of DNA methylation in CG context in inactive (winter) and active (summer) periods, related Figure 2.

| Protein ID | Protein name | Protein description | Ratio (iTRAQ) | Ratio (TMT) |
|------------|--------------|---------------------|---------------|-------------|
| Alsi17612  | LV001        | Ig lambda chain V region 4A | 1.38          | 1.42        |
| Alsi14324  | AF1L1        | Actin filament-associated protein 1-like 1 | 1.24          | 1.3         |
| Alsi15971  | FBLN3        | EGF-containing fibulin-like extracellular matrix protein 1 | 1.35          | 1.43        |
| Alsi19475  | KACB         | Ig kappa chain C region, B allele | 1.42          | 1.28        |
| Alsi05485  | SRCA         | Sarcalumenin | 2.93          | 2.21        |
| Alsi01963  | FCGBP        | IgGFc-binding protein | 1.76          | 1.89        |
| Alsi16621  | CAH6         | Carbonic anhydrase 6 | 1.72          | 1.48        |
| Alsi17112  | A1AT         | Alpha-1-antitrypsin | 0.67          | 0.56        |
| Alsi10652  | RABL6        | Rab-like protein 6 | 0.65          | 0.75        |
| Alsi14288  | VWF          | von Willebrand factor | 0.73          | 0.56        |
| Alsi13698  | VWA7         | von Willebrand factor A domain-containing protein 7 | 0.79          | 0.65        |
| Alsi01866  | A2ML1        | Alpha-2-macroglobulin-like protein 1 | 0.69          | 0.66        |
| Alsi23845  | ITIH3        | Inter-alpha-trypsin inhibitor heavy chain H3 | 0.64          | 0.6         |
| Alsi23375  | XPO2         | Exportin-2 | 0.53          | 0.78        |
| Alsi12372  | MCF2L        | Guanine nucleotide exchange factor DBS | 0.53          | 0.56        |
| Alsi26905  | KPYM         | Pyruvate kinase PKM | 0.28          | 0.34        |
| Alsi02255  | KPYR         | Pyruvate kinase PKLR | 0.56          | 0.64        |
| Alsi20997  | CLC11        | C-type lectin domain family 11 member A | 0.73          | 0.74        |
| Alsi17022  | RET4         | Retinol-binding protein 4 | 0.51          | 0.63        |
| Alsi04866  | COMP         | Cartilage oligomeric matrix protein | 0.65          | 0.51        |
| Alsi09977  | LEG4         | Beta-galactoside-binding lectin | 0.45          | 0.44        |
| Alsi21045  | APOH         | Beta-2-glycoprotein 1 | 0.47          | 0.39        |
| Alsi01416  | ACT5         | Actin, cytoplasmic type 5 | 0.64          | 0.79        |
| Alsi12373  | FA10         | Coagulation factor X | 0.51          | 0.56        |

# Winter/Summer
**SUPPLEMENTARY TEXT**

**Data summary**

We collected tissues from adult Chinese alligators in winter and summer and used multiple omics technologies (mRNA-Seq, BS-Seq, sRNA-Seq, iTRAQ, and TMT) (Figure 1, Table S1) to comprehensively explore the molecular mechanisms underlying reptile hibernation.

We produced 42 strand-specific mRNA-seq libraries, and generated 2,439 million paired-end reads, 88.36% of which were uniquely mapped to the Chinese alligator reference genome. Using these new transcriptome data as well as transcriptome data of Chinese alligator embryo (Lin et al., 2018), we annotated 27,500 protein-coding genes in the Chinese alligator genome.

Using tissues from female alligators, we produced 22 BS-seq libraries and generated 6,975 million clean read pairs (1,609 Gb of clean data), with an average depth of 16.11 per strand for each sample, and an average of 85.97% of genomic cytosines (Cs) being covered by at least five unique reads in each sample.

For sRNAs, we generated 129 million clean single-end reads from 10 sRNA-Seq libraries. 89.34% of the reads were 18–35 nt in length and were successfully mapped to the genome. We excluded other RNA species (rRNAs, tRNAs, snRNAs, snoRNAs), repetitive sequences, and transcript sequences, and with the aid of data from eight other tissues (testes and ovaries), we annotated 950 mature miRNAs. Among them, 132 miRNAs from 77 families were found in at least one other species and were thus identified as conserved miRNAs.
EXPERIMENTAL PROCEDURES

Sample sources and DNA and RNA extraction

Chinese alligator tissues (including liver, adipose, cardiac muscle, skeletal muscle, brain, hypothalamus, spleen, kidney, lung, small intestine, and thyroid tissues) were provided by the Changxing Yinjiabian Chinese Alligator Nature Reserve (CCANR) (Figure 1, Table S1). Winter samples were collected from two adult hibernating individuals (one male and one female) in January 2015, and summer samples were collected from two different adult individuals (one male and one female) during the breeding season, in May 2015. Serum samples for iTRAQ and TMT were collected from three female individuals in winter and summer, respectively. All samples were immediately stored in liquid nitrogen until use. Sample collection was performed with permission from the State Forestry Administration of China [Forest Conservation Permission Document (2014) 1545] and the Animal Ethics Committee of Zhejiang University (ZJU2015-154-13).

The gDNA used for BS-Seq and RNA used for mRNA-Seq was isolated from tissue samples using an AllPrep DNA/RNA Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer’s instructions. Total RNA for sRNA-Seq was isolated using a TRIzol RNA isolation kit (Invitrogen, Waltham, MA, USA) according to the manufacturer’s instructions.

Strand-specific cDNA library construction and sequencing

Three micrograms of RNA was used for strand-specific cDNA library construction using a NEBNext® Ultra™ Directional RNA Library Prep Kit for Illumina® (New England Biolabs, Ipswich, MA, USA), according to the manufacturer’s instructions. Library quality was evaluated on a Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA). The index-coded samples were clustered using a TruSeq PE Cluster Kit v3-cBot-HS on a cBot Cluster Generation System (Illumina Inc., San Diego, CA, USA). The library was sequenced on the Illumina HiSeq 2500 platform, generating 125-bp paired-end reads. In-house Perl scripts were used to preprocess the raw reads in fastq format. Reads containing adapter or poly-N sequences, and low-quality reads were filtered out. The Q20, Q30, and GC content of the remaining reads were calculated, and all subsequent analyses were based on these clean reads.
BS-Seq library construction and sequencing

An unmethylated λ DNA fragment was added to the gDNA to evaluate the bisulfite conversion efficiency for quality control of the bisulfite treatment. Six micrograms of gDNA and 30 ng λ DNA were mixed and fragmented into 200–300 bp by sonication. After end repair and acetylation, barcodes with methylated cytosines were added to the fragmented DNA. DNA bisulfite conversion was carried out twice using an EZ DNA methylation-Gold™ Kit (Zymo Research, Irvine, CA, USA). The DNA fragments were then amplified by PCR with KAPA Hifi HotStart Uracil + ReadyMix (Kapa Biosystems, Wilmington, MA, USA). DNA concentrations in the BS libraries were determined with a Qubit® 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA), and insert sizes were evaluated using the Bioanalyzer 2100 system. The index-coded samples were clustered using the TruSeq PE Cluster Kit v3-cBot-HS on the cBot Cluster Generation System, according to the manufacturer’s instructions. The BS library was sequenced on the Illumina HiSeq 2500 platform, generating 125-bp paired-end reads. In-house Perl scripts were used to preprocess the raw reads in fastq format. Low-quality reads, reads containing adapter and poly-N sequences, and reads shorter than 36 nt following adapter removal were filtered out. The Q20, Q30, and GC content of the remaining reads were calculated, and all subsequent analyses were based on these clean reads.

Small RNA library construction and sequencing

Three micrograms of total RNA was used to construct sRNA libraries using a NEBNext® Multiplex Small RNA Library Prep Set for Illumina® (New England Biolabs), according to the manufacturer’s instructions. The Agilent Bioanalyzer 2100 system was used for library quality assessment. The index-coded samples were clustered using the TruSeq SR Cluster Kit v3-cBot-HS on the cBot Cluster Generation System. After cluster generation, the sRNA libraries were sequenced on an Illumina HiSeq 2500 platform, generating 50-bp single-end reads.

RNA-Seq data analysis

The new transcriptome data produced in this study as well as transcriptome data of Chinese alligator embryo (Lin et al., 2018) were used to identify genes in Chinese alligator genome. An index of the Chinese alligator reference genome was built using Bowtie2 (Langmead and Salzberg, 2012),
and the cleaned reads were mapped to the reference genome using TopHat v. 2.0.12 (Kim et al., 2013). All aligned reads were assembled, and Cufflinks v. 2.1.1 was used to identify genes (Trapnell et al., 2010). TransDecoder and CPC were used to identify the coding region and protein-coding potential of each novel transcript (Haas et al., 2013; Kong et al., 2007). Genes with open reading frames larger than 150 bp and high protein-coding potential (score > 0) were subjected to further analysis. Transcription factor genes were identified by aligning all 27,500 genes to the animal transcription factor database TFDB2.0 (Zhang et al., 2015), using hmmsearch (Eddy, 2011).

Reads mapped to each gene were counted using HTSeq v. 0.6.1 (Anders et al., 2015), and differential gene expression between each pair of samples was analyzed using the DEGseq R package v. 1.12.0 (Mortazavi et al., 2008). Genes with an FDR < 0.005 and \(|\log_2(\text{fold change})| > 1\) were considered differentially expressed genes (DEGs). To correspond this with BS-Seq and sRNA-Seq data, we focused on the different season-biased transcriptomes of the female alligator for most of our analyses. However, we noted that gene expression patterns were largely similar in male and female non-gonadal tissues. The number of fragments per kilobase of exon per million mapped fragments (FPKM) was calculated to estimate gene expression levels. The WGCNA R package (Langfelder and Horvath, 2008) was used for weighted gene co-expression network analysis based on the RNA-Seq data to analyze correlations in expression patterns among Chinese alligator genes. Gene expression level heatmaps were constructed using TBtools (Chen et al., 2018).

BS-Seq data analysis

BS-Seq reads were aligned to the Chinese alligator reference genome using Bismark (v. 0.12.5) with default parameters (Krueger and Andrews, 2011). The Chinese alligator reference genome was transformed into fully BS-converted versions termed “T genome” (C-to-T converted) and “A genome” (G-to-A converted) and then indexed using Bowtie2 (Langmead and Salzberg, 2012). All cytosines of the BS-converted reads were transformed to thymines, and the reads were aligned to the “T genome.” All guanines of the BS-converted reads were transformed to adenosines and the reads were aligned to the “A genome.” The sequence reads that produced a unique best alignment from the two alignments (original Watson and Crick strand) were re-aligned to the original reference genome to infer the methylation state of all cytosines in sequence reads. Multiple reads mapped to
the same regions of genome were defined as clonal duplicates and were filtered out to avoid inaccuracy that might be caused by bias during PCR amplification. The BS library conversion rate was estimated as the percentage of cytosines sequenced at cytosine positions in the Λ reference genome.

To identify methylation sites, we modeled the sum of methylated cytosines (mCs) as a binomial (Bin) random variable with methylation rate (r), as mC ~ Bin (mC + umC*r).

The methylation level of each cytosine site was determined by the number of reads containing a methylated cytosine at the site of interest divided by the total number of reads covering the cytosine site. The methylation level of a specific region was calculated as the average methylation level of all cytosine sites in this region.

Differentially methylated regions (DMRs) between two samples were identified using swDMR (Wang et al., 2015). Since most of the mCs were in the CG context and the methylation levels in CHG and CHH contexts were low, we focused solely on CG sites for subsequent analyses. The sliding window was set to 1000 bp with a step length of 100 bp. To ensure statistical power, only windows with at least 10 CG sites and a coverage of 5 in each of the two compared samples were considered. Fisher’s exact test was employed and only windows with an FDR-adjusted \( p (q) < 0.05 \) and greater than two-fold methylation level change were considered DMRs. Genes containing DMRs in their putative promoter or/and gene body regions were defined as differentially methylated genes (DMGs).

**sRNA-Seq data analysis**

sRNA reads were cleaned to eliminate unqualified reads and then mapped to the Chinese alligator genome reference sequence using Bowtie v. 2.2.3 (Langmead and Salzberg, 2012) without allowing any mismatch. We then annotated the reference genome sequence using the Rfam database (Kalvari et al., 2018) and RepeatMaker (Smit et al., 2013-2015), and reads corresponding to rRNAs, tRNAs, snRNAs, snoRNAs, and repeat sequences, as well as exons and introns were removed. miREvo v. 1.1 (Wen et al., 2012) and mirdeep2 (Friedlander et al., 2012) were used to predict miRNAs through exploration of the secondary structure, the Dicer cleavage site, and the minimum
free energy of the reads. Predicted miRNAs were subjected to Rfam (Kalvari et al., 2018) for miRNA family analysis and identification of conserved miRNAs (found in at least one other species). In-house scripts were applied to obtain miRNA counts. Finally, the expression level of each miRNA was normalized as the number of transcripts mapped to the miRNA per million transcripts (TPM). DEGseq R package was used to analyze differential miRNA expression between paired samples (Wang et al., 2010). miRNAs with $q < 0.01$ and $|\log_2(\text{fold change})| > 1$ were assigned as DEmiRs. MiRanda, RNAhybrid, and PITA were used to predict target genes of miRNAs (Betel et al., 2010; Kertesz et al., 2007; Rehmsmeier et al., 2004). Target genes approved by at least one software package were considered targets. DESeq R package (Anders and Huber, 2010) was used to carry out principal component analysis (PCA) of sRNA-Seq data of the gonad samples and construct the plot.

**GO and KEGG enrichment analyses**

The GOseq 2.12 R package was used for GO enrichment analysis (Young et al., 2010). The KOBAS software package was employed for KEGG pathway enrichment analysis (Mao et al., 2005). GO terms and KEGG pathways with a $q < 0.05$ were regarded as significantly enriched.

**TMT/iTRAQ protein quantification and data analysis**

To reduce protein complexity and interference from highly abundant proteins, we used a ProteoMinerTM Kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer’s instructions to deplete high- abundance proteins in the serum samples. Protein concentrations were determined by Bradford protein assay (Bio-Rad Laboratories). Total protein (100 μg) was digested with Trypsin Gold (Promega, Madison, WI, USA) using a protein/trypsin ratio of 30:1 at 37 °C for 16 h. The peptides were dried by vacuum centrifugation and reconstituted in 0.5 M triethylammonium bicarbonate buffer and processed according to the manufacturer’s instruction for 8-plex iTRAQ (Applied Biosystems, Waltham, MA, USA) and 6-TMT labeling (Thermo Fisher Scientific). The labeled peptide mixtures were then pooled and dried by vacuum centrifugation. The peptides were separated by SCX chromatography using a LC-20AB HPLC Pump system (Shimadzu, Kyoto, Japan) and analyzed by LC-ESI-MS/MS analysis using a Q Exactive mass spectrometer (Thermo Fisher Scientific) coupled to the HPLC.
Peptides and proteins were identified by searching against the Chinese alligator database containing 27,500 sequences using the Mascot search engine v. 2.3.02 (Matrix Science, London, UK). Quantitative protein ratios were weighted and normalized by the median ratio in Mascot. We compared pairs of winter and summer samples. Only ratios with $p < 0.05$ and a fold change $> 1.2$ or $< 0.83$ in at least one pair were considered a significantly season-biased differentially expressed protein, and those with a different change trend in any pair were excluded.
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