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Osmotic Stress-induced Gene Expression in the Diencephalon, Kidney, Liver, and Breast Muscle of Chicks

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Endogenous water production is an important response for inducing water acquisition in birds, with proteins and lipids being major sources of endogenous water. However, the roles of protein and lipid metabolism-related gene expression in the regulation of their body fluid balance have not been investigated. This study aimed to clarify the roles of protein and lipid metabolism-related genes in osmoregulation in chicks. In Experiment 1, we examined the effects of 12 h of water deprivation on the mRNA levels of protein and lipid metabolism-related genes and feed intake in chicks. Feed intake was significantly decreased by water deprivation throughout the experimental period. The mRNA levels of vasotocin in the diencephalon were significantly increased by water deprivation. The mRNA levels of carnitine palmitoyltransferase 1A (CPT1A), the rate-limiting enzyme of mitochondrial fatty acid oxidation, were significantly increased by water deprivation in the liver, breast muscle, and diencephalon of the chicks. The mRNA levels of atrogin-1, a regulatory enzyme of the ubiquitin proteasome-system, were significantly increased by water deprivation in the breast muscle of the chicks. In contrast, the mRNA levels of fatty acid synthase, the rate-limiting enzyme of fatty acid synthesis, were significantly decreased by water deprivation in the liver of the chicks. In Experiment 2, the effects of intraperitoneal administration of hypertonic saline were examined under feed and water-deprived conditions. The mRNA levels of renal aquaporin 1, breast muscle atrogin-1, and diencephalon CPT1A were significantly increased 1 h after hypertonic saline injection. These results suggest that osmotic stress may induce protein catabolism in the skeletal muscle and fatty acid catabolism in the diencephalon of the chicks.

Key words: dehydration, drink, osmolality, sodium chloride

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Introduction

From the perspective of animal welfare, freedom from thirst has become one of the important requirements in the poultry industry[1,2]. In humans, increased blood osmolality is probably the strongest homeostatic signal for drinking[3]. In chickens, evidence suggests that the peripheral administration of angiotensin II, a hyperosmotic signal, induces thirst[4,5]. Clarification of the mechanism underlying osmotic regulation is necessary to further support the prevention of thirst in poultry production.

Osmotic stress induces antidiuresis in birds. Aquaporins (AQPs), a family of water-selective transport proteins, and the antidiuretic hormone vasotocin play critical roles in the conservation of water by producing hyperosmotic urine in kidneys[6,7]. In chicks, drinking hypertonic saline[8] and water deprivation[9] significantly increased the mRNA levels of vasotocin in the hypothalamus. Moreover, intraperitoneal administration of hypertonic saline significantly increased the mRNA levels of aquaporin (AQP)1 and 2 in the kidneys[10], and 24 h of water deprivation significantly increased the mRNA levels of AQP2 and 3 in the kidneys[10]. These findings suggest that changes in the mRNA levels of vasotocin and AQP1-3 reflect osmotic responses in chicks.

Endogenous water production plays a crucial role in water acquisition for animals in dehydrating environments, particularly in migrating birds[11]. Resident birds also produce endogenous water, the sum of metabolic water and water liberated from the catabolism of lean mass, from proteins and lipids. For example, the body fluid balance of fasting zebra finches without access to water appeared to be maintained by elevated fat catabolism[12]. Water restriction increases protein catabolism[13], including the oxidation of endogenous amino acids[14] in house sparrows as a means to maintain water balance. Interestingly, carnitine pal-

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mitoyltransferase 1A (CPT1A), the rate-limiting enzyme of mitochondrial fatty acid oxidation, plays an important role in the maintenance of whole-body fluid in mice[15]. However, the effects of osmotic stress on protein and lipid metabolisms have not been investigated in chickens.

In the present study, we examined the effects of water deprivation and intraperitoneal administration of hypertonic saline on the expression of protein and lipid metabolism-related genes in chicks. Our findings suggest that osmotic stress in chicks may influence protein and lipid catabolism in the skeletal muscle and diencephalon, respectively.

Materials and methods

Animals and diet

Day-old male chicks (White Leghorn, Julia Light) were purchased from local hatcheries (Japan Layer K.K., Gifu, Japan). They were given free access to water and a commercial chick starter diet (NICHIWA SANGYO Co., Ltd., Kobe, Japan). Chicks were reared in electrically heated cages under a 23-h/1-h light/dark cycle. The temperature was kept at 31 ± 2 °C. This study was approved by the Institutional Animal Care and Use Committee and conducted according to the Kobe University Animal Experimentation Regulation (2024–10-01).

Experiment 1. Effects of water deprivation on gene expression, serum components, and feed intake in chicks

Sugiura et al.[10] reported that 24 h of water deprivation significantly increased plasma sodium and renal AQPs mRNA levels in 7-day-old chicks. We found that 12 h of water deprivation significantly increased diencephalon vasotocin mRNA levels in 8-day-old chicks in a preliminary experiment (unpublished data). Based on these findings, the water deprivation period was set to 12 h in Experiment 1.

A total of twelve 5-day-old chicks were moved to individual cages (150 × 250 × 150 mm). Feed and water were supplied in a plastic feeder and waterer (78 × 58 × 77 mm). Room temperature was maintained at 25 ± 2 °C. After 3 days of acclimatization, chicks were weighed and divided based on body weight. Chicks in the control group were given feed and water for 12 h. Chicks in the water deprivation group were given feed without water for 12 h. Feed intake and water intake were measured every 3 h. At the end of the experiment, the chicks were euthanized by decapitation. Blood was collected from the carotid artery and kept at room temperature for 60 min. Then, the serum was separated by centrifugation at 1,900 ×g for 15 min at 4 °C.

Serum components analyses

The serum concentrations of sodium were measured using the atomic absorption method (Z-2010, Polarized Zeeman Atomic Absorption Spectrophotometer, Hitachi, Tokyo, Japan) according to the supplier's recommendations. The serum concentrations of glucose were measured using a commercial kit (LabAssay^{TM} Glucose, Code: 291-94001, Fujifilm Wako Pure Chemicals Co., Osaka, Japan).

Real-time PCR analysis

The diencephalon, kidney, and pieces taken from the center of the liver and breast muscle were preserved in RNAlater® (Sigma-Aldrich, St. Louis, MO, USA). Total RNA was extracted from the tissues using Sepazol-RNA I Super G (Nacalai Tesque, Inc., Kyoto, Japan). First-strand cDNA was synthesized from total RNA using Rever Tra Ace® qPCR RT Master Mix with gDNA Remover (Toyobo Co., Ltd., Osaka, Japan). The levels of mRNA were quantified for each primer using TB Green Premix Ex Taq II (Tli RNaseH Plus, Takara Bio Inc., Otsu, Japan) according to the supplier's recommendations, using the Thermo Scientific Piko Real Real-Time PCR System (Thermo Fisher Scientific, Vantaa, Finland). Primer sequences are shown in Table 1. The mRNA levels of target genes were normalized to those of RPS17.

Experiment 2. Effects of intraperitoneal hypertonic saline administration on gene expression and serum components in chicks

Sugiura et al.[10] reported that intraperitoneal injection of 3M NaCl in chicks significantly increased blood sodium and renal AQP1 mRNA levels 1 h after injection in chicks. Therefore, we conducted our experiment under the same conditions.

A total of twelve 8-day-old chicks were weighed and divided into two groups based on body weight; the groups received saline (0.15 mol/L sodium chloride solution) or hypertonic saline (3 mol/L sodium chloride solution) intraperitoneally (5 mL/kg body weight). After 1 h of feed and water deprivation, chicks were euthanized by decapitation. The blood, diencephalon, kidney, liver, and breast muscle were collected, and the serum components and mRNA levels of target genes were analyzed, as described above.

Data analyses

The means of mRNA and serum component levels in experiments 1 and 2 were analyzed using the Student's *t*-test and Microsoft Excel 2016 (Microsoft Corporation, Redmond, WA, USA). The means of feed intake in Experiment 1 were analyzed by two-way analysis of variance (ANOVA). When a significant interaction was detected, the data were analyzed by Tukey Kramer's test for multiple comparison. A significant difference was defined as $P < 0.05$.

Results

In Experiment 1, the mRNA levels of vasotocin in the diencephalon were significantly increased by water deprivation (Fig. 1A). The mRNA levels of AQP2 in the kidney were also increased by water deprivation, but the difference was not significant (Fig. 1B, $P = 0.07$). These results suggest that 12 h of water deprivation induced osmotic stress in chicks. The mRNA levels of CPT1A were significantly increased by water deprivation in the liver, breast muscle, and diencephalon of chicks (Figs. 1A, C, and D). The mRNA levels of atrogin-1, a regulatory enzyme of the ubiquitin proteasome-system, were significantly increased by water deprivation in the breast muscle of chicks (Fig. 1D). In contrast, the mRNA levels of fatty acid synthase, the rate-limiting enzyme of fatty acid synthesis, were significantly decreased by water deprivation in the liver of chicks (Fig. 1C). Considering that a significant interaction was detected between feed intake and time ($P < 0.01$), the data were analyzed by multiple compari-

Table 1. **Primer sequences for real-time PCR analysis**

Gene		Nucletotide sequence	GenBank Accession Number
AVT	Sense	5'-TCC GGG CAC ACT CAG CAT-3'	X55130
	Antisense	5'-ATG TAG CAG GCG GAG GAC AA-3'	
AQP1	Sense	5'-GGG ACA TCT CCT TGC AAT TGA TTA C-3'	NM_001039453
	Antisense	5'-TGA TTG GGC CAA CCC AGA AGA-3'	
AQP2	Sense	5'-GGC CAT CAA CAA GCT GCA TAA-3'	NM_001292072
	Antisense	5'-GAC CCC ATG TTG TCC TCC CT-3'	
AQP3	Sense	5'-TTT GGG CTC TAC CAT GAT GCC-3'	XM_424500
	Antisense	5'-TGT TGA GAT GGG TAG GTG GC-3'	
CPT1A	Sense	5'-GCC CTG ATG CCT TCA TTC AA-3'	NM_001012898
	Antisense	5'-ATT TTC CCA TGT CTC GGT AGT GA-3'	
CPT1B	Sense	5'-GCC ATC GAC GCG TCC TT-3'	DQ314726
	Antisense	5'-AAG CAG CAG AAA TCG ACG TCA T-3'	
FASN	Sense	5'-ACT GTG GGC TCC AAA TCT TCA-3'	J03860
	Antisense	5'-CAA GGA GCC ATC GTG TAA AGC-3'	
NPY	Sense	5'-CTT GTC GCT GCT GAT CTG-3'	NM_205473
	Antisense	5'-GCC TCA GAG CCG AGT AGT-3'	
POMC	Sense	5'-AGA TGG AGA AGG GTT GGA A-3'	NM_001031098
	Antisense	5'-CGT TGG GGT ACA CCT TGA-3'	
Atrogin-1	Sense	5'-CAC CTT GGG AGA AGC CTT CAA-3'	NM_001030956
	Antisense	5'-CCG GGA GTC CAG GAT AGC A-3'	
LC3B	Sense	5'-TCC GAG ATC AGC ATC CAA CT-3′	NM_001031461
	Antisense	5'-CAC CAT GCT GTG TCC GTT C-3'	
RPS17	Sense	5'-GCG GGT GAT CAT CGA GAA GT-3'	NM_204217
	Antisense	5'-GCG CTT GTT GGT GTG GAA GT-3'	

AVT, vasotocin; AQP, aquqporin; CPT, carnitinepalmitoyltransferase; FASN, fatty acid syntase; NPY, neuropeptide Y; POMC, proopiomelanocortin; LC3B, microtubule-associated protein 1 light chain 3 isoform B; RPS, ribosomal protein

sons. As a result, feed intake was significantly decreased after 3 h of water deprivation (Fig. 2). No significant change was observed in serum components (Table 2). These results suggest that osmotic stress and/or a decrease in feed intake influence the expression of protein and lipid metabolism-related genes in chicks.

We next examined the effects of hypertonic saline injection on the mRNA levels of osmolality-, protein metabolism-, and lipid metabolism-related genes in chicks under feed- and water-deprived conditions. Intraperitoneal injection of hypertonic saline significantly increased the mRNA levels of renal AQP1 (Fig. 3B) and the serum sodium concentration in chicks (Table 3). The mRNA levels of AQP2 were also increased by hypertonic saline injection, but the difference was not significant (Fig. 3B, $P = 0.09$). These results suggest that osmotic stress was induced by hypertonic saline injection. Moreover, the mRNA levels of atrogin-1 in the breast muscle of chicks were significantly increased by hypertonic saline injection (Fig. 3D). Notably, the mRNA levels of CPT1A in the diencephalon were significantly increased by hypertonic saline injection (Fig. 3A), but the mRNA levels of CPT1A were not significantly changed in the liver or breast muscle (Figs. 3C and D). These results suggest that hypertonic saline induces osmotic stress in chicks and may induce protein catabolism in the skeletal muscle and fatty acid catabolism in the diencephalon.

Discussion

Both water deprivation and hypertonic saline injection significantly increased atrogin-1 mRNA levels in the breast muscle of the chicks, suggesting that proteolysis is induced by osmotic stress in chicks. Protein is one of the sources of endogenous water in migrating birds[11,12] and resting birds[13,14]. The induction of protein catabolism in skeletal muscle may decrease meat yield in meat-type chickens. Therefore, freedom from thirst may be an important requirement in the poultry industry not only for animal welfare but also for meat production.

In Experiment 1, water deprivation significantly influenced the mRNA levels of protein and lipid metabolism-related genes, as well as the feed intake, in chicks. We previously showed that fasting significantly increases the mRNA levels of CPT1A and decreases the mRNA levels of FASN in the chicken liver[16], and the mRNA levels of atrogin-1 in breast muscle[17]. Therefore, the significant changes observed in the liver and breast muscle

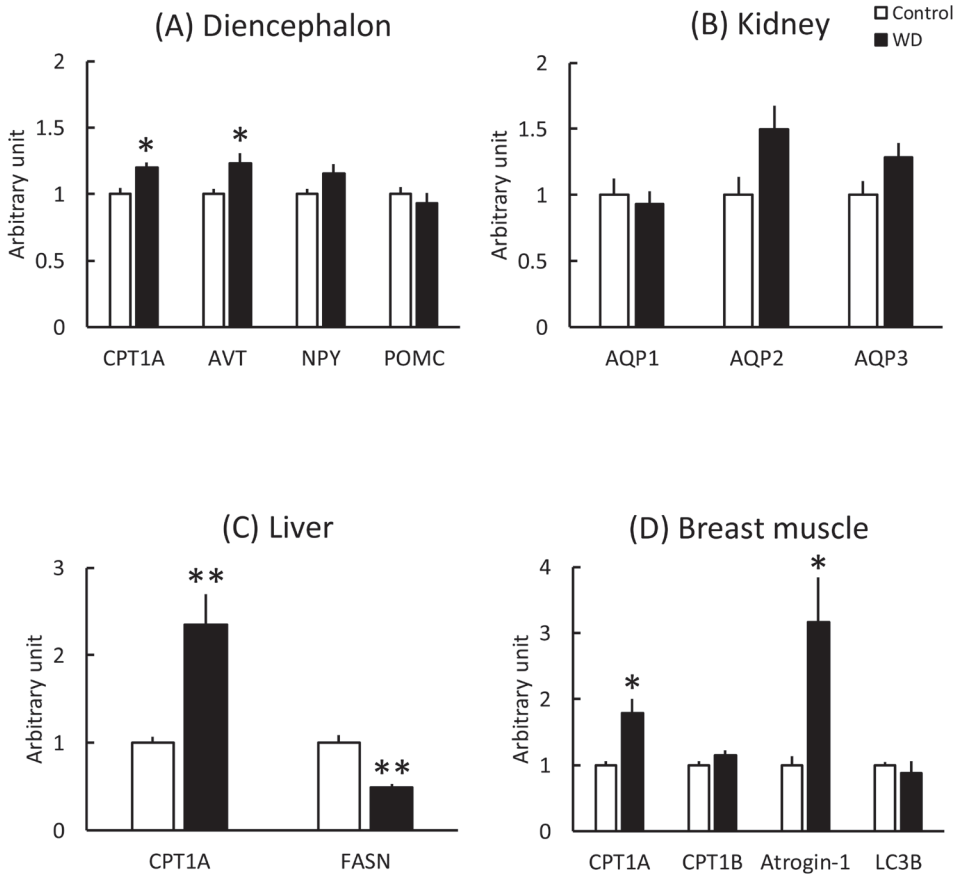

Fig. 1. **Effects of water deprivation on gene expression in the diencephalon (A), kidney (B), liver (B), and breast muscle (D) of chicks**

Data are expressed as the mean ± SEM of six replicates in each group. The asterisk indicates significance with respect to the control group (*, $P < 0.05$; **, $P < 0.01$). WD, water deprivation.

Table 2. **Effects of water deprivation on serum components in chicks**

	Control	Water deprivation
Sodium (mmol/L)	136 ± 3	138 ± 2
Glucose (mg/100 mL)	243 ± 5	248 ± 5

Values are mean ± SEM for six replicates in each group.

in Experiment 1 may have been induced by the decreased feed intake of the chicks. However, the effect of dehydration or interaction between decreased feed and water intake may be involved in the significant changes observed in Experiment 1. Investigating the effects of water deprivation under fasting or pair-feeding conditions remains necessary.

Evidence suggests that hypothalamic CPT1A is involved in appetite regulation in mammals. The long-term expression of a permanently activated CPT1A isoform in the hypothalamus triggers hyperphagia, causing excessive gain of weight[18]. In

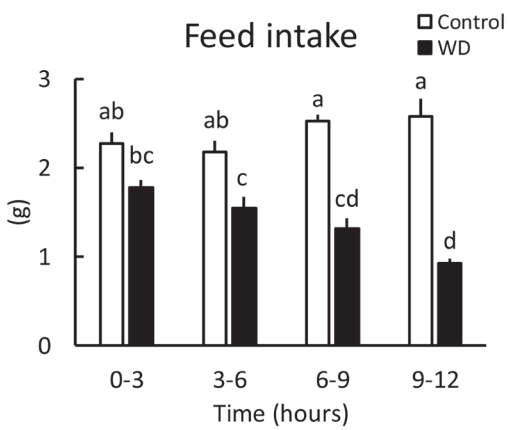

Fig. 2. **Effects of water deprivation on feed intake in chicks**

Data are expressed as the mean ± SEM of six replicates in each group. Groups with different letters are significantly different ($P < 0.05$). WD, water deprivation.

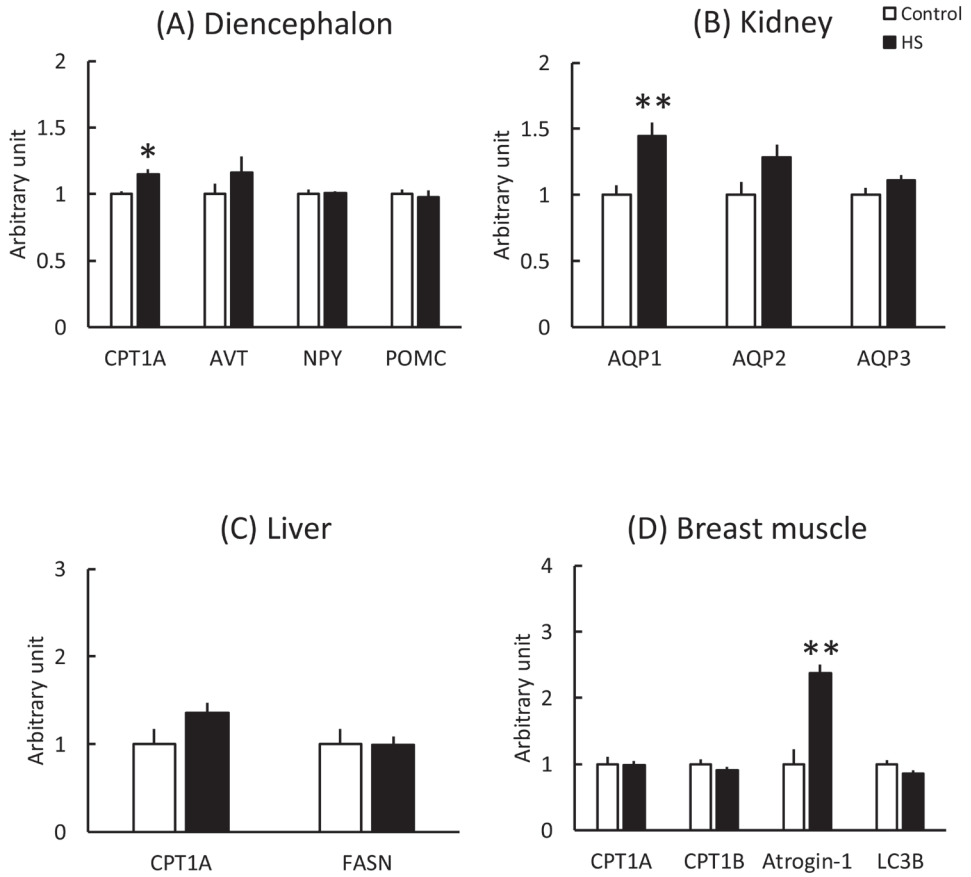

Fig. 3. **Effects of intraperitoneal hypertonic saline injection on gene expression in the diencephalon (A), kidney (B), liver (C), and breast muscle (D) of chicks**

Data are expressed as the mean ± SEM of six replicates in each group. The asterisk indicates significance with respect to the control group (*, $P < 0.05$; **, $P < 0.01$). HS, hypertonic saline.

Table 3. **Effects of hypertonic saline injection on serum components in chicks**

	Control	Hypertonic saline
Sodium (mmol/L)	137 ± 2	160 ± 4*
Glucose (mg/100 mL)	254 ± 4	270 ± 13

Values are mean ± SEM for six replicates in each group.

contrast, central administration of CPT1 inhibitor increases long chain acyl-CoA in the hypothalamic arcuate nucleus and decreases food intake in rats[19]. In addition, a recent finding suggested that CPT1A in hypothalamic Agouti-related protein (AgRP) neurons plays a role in the neuronal processes necessary for the maintenance of whole-body fluid[15]. In the present study, hyperosmotic saline injection significantly increased the mRNA levels of CPT1A in the diencephalon of chicks. Further study is needed to examine whether central injection of a transcription factor of CPT1, such as WY14643, influences whole-body fluid balance in chicks.

The molecular mechanism underlying the hypertonic saline injection-induced upregulation of diencephalon CPT1A and breast muscle atrogin-1 in chicks is not clear. However, osmotic stress responses are complex and observed in most living organisms, from yeast to mammals[20]. For example, exposure to osmotic stress induced the expression of more than 120 genes that were mostly dependent on p38 MAPKs, the mammalian orthologues of yeast Hog1, in mice embryonic fibroblasts[21]. Osmotic change is directly sensed by the mitochondria, activates aerobic glycolysis, and produces ATP quickly, which may contribute to cell survival under hyperosmotic conditions[22], although the transcriptional changes in CPT1A were only found in the diencephalon. Species- and tissue-specific mechanisms require further investigations.

In the present study, dehydration and hypertonic saline injection showed different effects on the expression of several genes. For example, the mRNA levels of hepatic FASN and CPT1A were not influenced by hypertonic saline injection. Liver is the primary organ of fatty acid synthesis, and liver and skeletal mus-

cles are major organ of fatty acid oxidation in chicks. Therefore, the changes in the mRNA levels of hepatic FASN and CPT1A were likely because of the decreased feed intake. Significant increase of renal AQP1 mRNA levels was observed only in Experiment 2. Sugiura et al.[10] reported that hypertonic saline injection significantly increased renal AQP1 mRNA levels, whereas 24 h of water deprivation significantly increased renal AQP2 and 3 mRNA levels in chicks. AQP1 is located in the proximal tubule of kidneys in quails, whereas AQP2 and AQP3 are present in the collecting duct[7]. Therefore, the physiological roles of AQP1-3 appear to vary between osmotic stress conditions. Serum sodium concentration was significantly increased only in Experiment 2. The reason for no changes in serum sodium concentration in Experiment 1 is not clear, but one possible explanation is that dietary sodium intake may have been decreased in Experiment 1. Thus, although the detail mechanism is not clear, different responses in gene expression between experiments 1 and 2 may be physiologically reasonable responses in chicks.

Post-hatch food and water deprivation negatively influence development, performance, and welfare of chickens[23]. Therefore, physiological and molecular mechanisms involved in the regulation of food intake in neonatal chicks have been investigated in recent decades[24–26]. However, the regulatory mechanisms involved in the regulation of water intake in chicks have not yet been investigated. We recently found that hypothalamic neuropeptides may be involved in water intake by 8-day-old chicks[27]. Evidence suggests that vasotocin and AQPs are involved in the osmotic regulation of 7-day-old chicks[10]. Therefore, we focused on the role of protein and lipid metabolism-related gene expression in osmotic regulation in 8-day-old chicks. However, further studies are required to investigate the osmoregulation system in more mature chickens in the future.

In this study, we found that osmotic stresses not only induce osmoregulatory gene expression in the kidneys but also induce atrogin-1 gene expression in the breast muscle and CPT1A gene expression in the diencephalon of chicks. Our findings suggest that the appropriate regulation of osmolality in chickens helps to quench thirst while also improving meat production in the poultry industry.

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Author contributions

Yuhui Zhang and Kaoruko Murata conducted the experiments and analyzed the data; Kazuhisa Honda and Takaoki Saneyasu designed the experiments; Yuhui Zhang wrote the manuscript; Junya Takegaki, Takaoki Saneyasu, and Kazuhisa Honda edited the manuscript.

Conflict of interest

The authors declare no conflict of interest.

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