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ORIGINAL PAPER

Seasonal flexibility of the gut structure and physiology in *Eremias multiocellata*

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Abstract

Although gut seasonal plasticity has been extensively reported, studies on physiological flexibility, such as water-salt transportation and motility in reptiles, are limited. Therefore, this study investigated the intestinal histology and gene expression involved in water-salt transport (*AQP1*, *AQP3*, *NCC*, and *NKCC2*) and motility regulation (*nNOS*, *CHRM2*, and *ADRB2*) in desert-dwelling *Eremias multiocellata* during winter (hibernating period) and summer (active period). The results showed that mucosal thickness, the villus width and height, the enterocyte height of the small intestine, and the mucosal and submucosal thicknesses of the large intestine were greater in winter than in summer. However, submucosal thickness of the small intestine and muscularis thickness of the large intestine were lower in winter than in summer. Furthermore, *AQP1*, *AQP3*, *NCC*, *nNOS*, *CHRM2*, and *ADRB2* expressions in the small intestine were higher in winter than in summer; *AQP1*, *AQP3*, and *nNOS* expressions in the large intestine were lower in winter than in summer, with the upregulation of *NCC* and *CHRM2* expressions; no significant seasonal differences were found in intestinal *NKCC2* expression. These results suggest that (i) intestinal water-salt transport activity is flexible during seasonal changes where *AQP1*, *AQP3* and *NCC* play a vital role, (ii) the intestinal motilities are attenuated through the concerted regulation of *nNOS*, *CHRM2*, and *ADRB2*, and (iii) the physiological flexibility of the small and large intestine may be discrepant due to their functional differences. This study reveals the intestinal regulation and adaptation mechanisms in *E. multiocellata* in response to the hibernation season.

Keywords Lizard · Hibernation · Intestine · Water-salt balance · Motility

Abbreviations

ADRB2	Adrenoceptor beta 2
AQPs	Aquaporins
CHRM2	Cholinergic receptor muscarinic 2
ENS	Enteric nervous system
NCC	Na^+-Cl^- cotransporter
NKCC2	Na^+-K^+-$2Cl^-$ cotransporter
NO	Nitric oxide
nNOS	Neuronal nitric oxide synthase

Introduction

The digestive tract is an important organ for energy supply, growth, survival, and reproduction in animals (Karasov et al. 2011), and it has been demonstrated to be one of the most responsive organs to environmental conditions (Pennisi 2005). Numerous studies have revealed that the digestive tract needs flexibility to meet energy demands as well as the challenges of the environment, diet, and predators (Bozinovic et al. 1990; Pennisi 2005; Piscitiello et al. 2020). Digestive flexibility is the induced modification of a morphological or physiological trait in response to changes in the environment, of which both types of traits are closely related to each other. However, most of our understanding of digestive flexibility is based on a few morphological and structural features, and the aspect of physiological flexibility, such as the regulation of water-salt balance and motility, needs to be further explored.

The intestine is responsible for the digestion and absorption of food and water, and is considered to play an indispensable role in maintaining the body's water-salt balance,

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which requires the daily transport of a large amount of fluid. On average, all secreted and ingested water is absorbed by the small and large intestines at approximately 84 and 16%, respectively (Laforenza 2012). Therefore, water transport by the intestinal epithelium is of considerable physiological importance.

Aquaporins (AQPs) are selectively distributed in epithelial cells and play a major role in the transport of water, solutes, ions, electrolytes, proteins, and nucleic acids between intracellular and extracellular fluids (Masyuk et al. 2002; Laforenza 2012; Brown 2017). Particularly, AQP1 and AQP3 expressed in the epithelial cells of the small and large intestines play an important role in the transcellular water transport (Gallardo et al. 2002; Masyuk et al. 2002). The secretion and absorption of electrolytes are also essential functions of the intestine, and the Na^+-Cl^- (NCC) and Na^+-K^+-$2Cl^-$ cotransporters (NKCC2) are crucial in maintaining cellular water and electrolyte contents and promoting salt and water movement across polarized cells (Djurisic and Forbush 2006; Lionetto and Schettino 2006). NCC are involved in salt and calcium absorption in the intestine (Bazzini et al. 2005), and NKCC2 are involved in the absorption of sodium, potassium, and chloride ions from the intestinal lumen (Ando et al. 2003; Cutler and Cramb 2008; Vistro et al. 2019).

The enteric nervous system (ENS) autonomously regulates gastrointestinal functions such as motility, mucosal secretion and absorption, blood flow, the epithelial barrier, and epithelial proliferation and differentiation (Furness 2012). Acetylcholine (ACh) is the main excitatory neurotransmitter of the gastrointestinal tract's smooth muscle, whereas nitric oxide (NO), as an inhibitory neurotransmitter, is responsible for the inhibition of this muscle (Mazet 2014). NO is released through neuronal nitric oxide synthase (nNOS) activity and, apart from regulating secretion and resorption, it has been demonstrated to be crucial for smooth muscle relaxation and motility in the stomach and small and large intestines (Holzer et al. 2001; Gallego et al. 2016). In contrast, ACh may cause the excitation of gastrointestinal smooth muscle by activating muscarinic receptors. Cholinergic receptor muscarinic 2 (CHRM2) is widely distributed in smooth muscles throughout the body, including the gastrointestinal tract, where it plays a major role in maintaining systemic smooth muscle contraction (Uchiyama and Chess-Williams 2004). In addition, as a major receptor in smooth muscles, adrenoceptor beta 2 (ADRB2) is expressed in both the small and large intestines (Johnson 2006). The adrenaline binding to ADRB2 induces smooth muscle relaxation (Kamiar et al. 2021).

The changes in ambient temperature and food availability drive the seasonal acclimatization of phenotypic flexibility in the digestive tract (Naya et al. 2008; Liu et al. 2013; Ma et al. 2018). Hibernation is correlated with great physiological flexibility that allows animals to adjust energy acquisition, storage, and expenditure processes according to current environmental conditions (Naya et al. 2008). However, studies evaluating the flexibility of gut structure and physiology in hibernating reptiles are relatively scarce. As poikilotherms, lizards are highly sensitive to external conditions. *Eremias multiocellata* is a small-sized, omnivorous, and viviparous lizard primarily distributed in desert and semi-desert regions with low water and food availability. It has been shown that phenotypic plasticity may be critical for *E. multiocellata* to effectively respond to climate change (Ma et al. 2018; Zhong and Wang 2022). In this study, we investigated seasonal intestinal flexibility, including structure and regulation of water-salt transport and motility, in *E. multiocellata*. Considering the critical role of AQPs, $Na^{(+)}$ transporters, NO, CHRM2, and ADRB2, we evaluated the intestinal histology and gene expressions of *AQP1*, *AQP3*, *NCC*(*SLC12A3*), *NKCC2*(*SLC12A1*), *nNOS* (*NOS1*), *CHRM2*, and *ADRB2* in this species during winter and summer.

Materials and methods

Animals

Adult *E. multiocellata* were seasonally collected by hand on the sands of the Baijitan National Nature Reserve (37°59′35.7″N, 106°21′39.8″E; elevation 1900 m) in Ningxia Province, northern China. This area is a desert with an average annual precipitation of 206.2–255.2 mm. The mean temperature is highest from June to July (average: 23.94 °C; relative humidity 42%) and lowest from December to January (average: − 5.7 °C; relative humidity 43%). *E. multiocellata* is highly active during the summer months (i.e., May to July), and activity is greatly reduced during autumn in September. From October to April, they hibernate. After collection, the lizards were transferred to the laboratory on the day of capture and housed in cages with sand in a room at natural temperature and photoperiod. Food (mealworms) and water were provided ad libitum. The lizards were divided into two groups: the summer (active) group ($n = 11$, male) and winter (hibernated) group ($n = 12$, male). The summer group was captured and sacrificed in June. The winter group was captured over a few-day period just before entering hibernation. Laboratory observations indicate that the lizards became continuously inactive and foraging activity stopped when entering hibernation (in October), regardless of food and water availability. The winter group was sacrificed while torpid in December. The lizards were maintained and treated in accordance with the guidelines for animal care established by the National Institutes of Health (Bethesda, MD, USA), using protocols

approved by the Animal Care and Use Committee of North Minzu University.

Histological examination

Each individual's body mass was measured using an electronic balance. The animals were anesthetized with an intraperitoneal injection of 2 mL ethyl carbamate (20%) and sacrificed through decapitation. Subsequently, the small and large intestines (summer, $n=5$; winter, $n=5$) were excised and washed with physiological saline to remove food residues, carefully dried with absorbent paper and weighed. After weighing,they were fixed with 4% paraformaldehyde for 48 h for histological staining. For molecular assays, the intestine (summer, $n=6$; winter, $n=7$) was immediately frozen in liquid nitrogen and stored at − 80 °C until subsequent RNA isolation. After 48 h of fixation, the intestines were dehydrated in a graded ethanol series (70, 80, 90, 95, and 100%), cleared in xylene, embedded in paraffin, and sectioned to a thickness of 4 µm. After deparaffinization, the sections were stained with hematoxylin and eosin.

Histological variables were measured using a Motic Microimaging System (Motic China Group Co., Ltd., Nanjing, China), and the slides were randomized and coded such that sample group designation was unknown to the observer. Individual intestinal parameters in each group were measured, including (1) mucosa, submucosa, and muscularis thickness—the muscularis included the circular and longitudinal muscle layers; (2) enterocyte height and diameter; (3) villus height and width; and (4) crypt depth (Naya et al. 2009a; Bo et al. 2018). Finally, the sections were imaged using the Motic Microimaging System.

Intestinal gene expression

Total RNA from the small and large intestines was extracted using an RNA extraction kit (Takara Bio Inc., Shiga, Japan) according to the manufacturer's instructions. The isolated RNA was quantified using an ultra-micro spectrophotometer (Maestro NanoPro MN-913A; MaestroGen Inc., Norcross, GA, USA), and purity was evaluated based on the OD_{260}/OD_{280} absorption ratio. Moreover, integrity was confirmed via agarose gel electrophoresis, after which reverse transcription was performed according to the instructions provided with the cDNA synthesis kit (Takara Bio Inc.).

Primers were designed using OLIGO Primer Analysis Software v. 7 (OLIGO, Colorado Springs, CO, USA), based on the relevant sequences published in the GenBank database for the "common lizard (*Zootoca vivipara*)," "sand lizard (*Lacerta* agilis)," and "common wall lizard (*Podarcis muralis*)" (Table 1). All primers were synthesized by Shanghai Sangon Biological Co. (Shanghai, China). The reaction mixture (50 µL) of target genes contained 2.5 µL cDNA, 1.5 µL forward primer, 1.5 µL reverse primer, 25 µL 2 × M5 HiPer plus Taq HIFI PCR (Mei5 Biotechnology Co. Ltd., Beijing, China), and 19.5 µL ddH_2O. Amplification was conducted under the following conditions: pre-denaturation at 95 °C for 3 min, followed by 36 cycles of denaturation at 94 °C for 25 s, annealing at 55 °C for 25 s, and extension at 72 °C for 10 s. The reaction mixture (50 µL) of *β*-actin contained 2 µL cDNA, 2 µL forward primer, 2 µL reverse primer, 5 µL 10 × Ex Taq Buffer (Mg^{2+}) (20 mM) (Takara Bio Inc.), 4 µL dNTP Mixture, 0.25 µL TaKaRa Ex Taq (Takara Bio Inc.), and 34.75 µL ddH_2O. Amplification was conducted under the following conditions: pre-denaturation at 95 °C for 3 s, followed by 30 cycles of denaturation at 95 °C for 5 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s. PCR product specificity and purity were evaluated via 2% gel electrophoresis; nevertheless, only the expected amplification bands are shown in the gel images, and no other bands were visible. Moreover, sample cycle threshold (CT) values were normalized to the CT values of 18S RNA, and to confirm the amplification of target genes by PCR, the amplified products were sequenced and analyzed (Shanghai Sangon Biological Co). The products showed high homology with the target genes from the lizard species used to design the primers (i.e., common, sand, and common wall lizards).

Finally, the expression levels of target genes were determined using a real-time fluorescence quantitative PCR instrument (qt2.2; Analytical Instruments GmbH, Jena, Germany). The reaction mixture (25 µL) contained 1 µL cDNA, 1 µL forward primer, 1 µL reverse primer, 12.5 µL TB Green

Table 1 Primers used in the PCR test

Target gene	Primer sequence (5′-3′)	Product length (bp)
AQP1	F: GAATCAATCCAGGACAAGGCC	202
	R: GACCCGAAAGATCTTGCAGG	
AQP3	F: TGACCAGTTCATCGGCACA	100
	R: AACGGTGAAAGCCTCCAGT	
NCC(*SLC12A3*)	F: ACCATCATTCCAGCCAGTGA	198
	R: CACGGCAGGATCCTTCAAAT	
NKCC2(*SLC12A1*)	F: AGGACTGGAAAGAAGCCACC	221
	R: GCCGCCATTTCCTTCTTCAA	
nNOS (*NOS1*)	F: CTCCTCCCCTGACATGTACC	162
	R: GGAGCTCCTCTGACAAAACA	
CHRM2	F: ACCGTGGAGATTGTAGGTCC	175
	R: TCCAGGTAATGATGAAGGCCA	
ADRB2	F: CGTCATCCCCAAATTTGTGTACA	157
	R: TCCATTGACGTACATTTTCAGGG	
β-actin	F: GCCAAGTGATCACCATCGG	107
	R: GTTGAAGGTGGTTTCGTGGA	

Premix Ex Taq II, and 9.5 µL ddH_2O. Amplification was conducted under the following conditions: pre-denaturation at 95 °C for 30 s, followed by 40 cycles of denaturation at 95 °C for 5 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s. Subsequently, a melt curve analysis was performed to verify PCR specificity, with the housekeeping gene *β-actin* as the endogenous control. Expression ratios relative to *β-actin* were calculated.

Data analysis

Statistical analyses were performed using SPSS, version 20.0 (SPSS Inc., Chicago, IL, USA). Differences in body and intestinal mass, intestinal histology parameters, and target gene expression between winter and summer were compared using independent *t* tests. Data were expressed as the mean ± standard error (mean ± SE), and statistical significance was considered at $P < 0.05$.

Results

Intestinal mass and histomorphology

Independent *t* tests showed that body mass of *E. multiocellata* was significantly lower in winter than in summer ($t_{(21)} = 4.088$, $P = 0.002$, Cohen's d = 1.74). The relative small intestine mass ($t_{(21)} = 2.483$, $P = 0.022$, Cohen's d = 1.035), and relative large intestine mass of ($t_{(21)} = 3.869$, $P = 0.001$, Cohen's d = 1.634) were significantly higher in winter than in summer; however, no significant differences were observed in the mass of the small ($t_{(21)} = 0.943$, $P = 0.357$, Cohen's d = 0.393) or large intestines ($t_{(21)} = 1.806$, $P = 0.096$, Cohen's d = 0.767) (Table 2).

The small intestine of *E. multiocellata* comprised the mucosa, submucosa, muscularis, and serosa. The muscularis consisted of an inner ring and outer longitudinal muscles. Microscopically, longer finger-like villi and intestinal glands were evident, and the structure of the villi was clear. The villi were composed of a single layer of columnar epithelium, as well as absorptive and goblet cells in the lamina propria, and the villi were longer and more densely arranged in winter.

Table 2 Body mass, intestinal mass, and relative intestinal mass of *E.multiocellata* during summer and winter

	Summer (n = 11)	Winter (n = 12)
Body mass (g)	7.432 ± 0.736	4.371 ± 0.139*
Small intestine mass (g)	0.053 ± 0.0039	0.0478 ± 0.0034
large intestine mass (g)	0.066 ± 0.0068	0.0538 ± 0.0022
Relative small intestine mass (g)	0.0077 ± 0.0008	0.0105 ± 0.0074*
Relative large intestine mass (g)	0.0093 ± 0.0007	0.0123 ± 0.0004*

Values are expressed as Means ± SE. Asterisk (*) indicate significant difference ($P < 0.05$)

An independent *t* test showed that the mucosa thickness of the small intestine ($t_{(8)} = 2.82$, $P = 0.022$, Cohen's d = 1.789) was greater in winter than in summer, and the villus width ($t_{(8)} = 4.395$, $P = 0.009$, Cohen's d = 2.78) and height ($t_{(8)} = 5.012$, $P = 0.001$, Cohen's d = 3.17), as well as the enterocyte height ($t_{(8)} = 7.975$, $P < 0.001$, Cohen's d = 5.044) in the small intestine, were significantly increased in winter compared to those in summer. However, the thickness of the submucosa in the small intestine was lower in winter than in summer ($t_{(8)} = -3.946$, $P = 0.014$, Cohen's d = − 2.496), and no significant seasonal differences were found in muscularis thickness ($t_{(8)} = -1.765$, $P = 0.0152$, Cohen's d = − 1.116), crypt depth ($t_{(8)} = -0.429$, $P = 0.679$, Cohen's d = − 0.272), or enterocyte diameter ($t_{(8)} = -0.294$, $P = 0.779$, Cohen's d = − 0.186) (Fig. 1, Table 3).

The structure of the large intestine in *E. multiocellata* was similar to that of the small intestine, and a clear intestinal fold fissure structure could be observed microscopically. The mucosal epithelium was a simple column epithelium with goblet cells in the lamina propriai, and the serosa contained a large number of fat cells. An independent *t* test showed that mucosa ($t_{(8)} = 4.053$, $P = 0.004$, Cohen's d = 2.564) and submucosa ($t_{(8)} = 4.514$, $P = 0.002$, Cohen's d = 2.855) thickness, as well as the enterocyte height ($t_{(8)} = 0.5.746$, $P < 0.001$, Cohen's d = 0.415), were significantly larger in winter than in summer. In contrast, muscularis thickness in the large intestine was significantly lower in winter than in summer ($t_{(8)} = -5.815$, $P < 0.001$, Cohen's d = − 3.678), and the enterocyte diameter showed no significant seasonal differences ($t_{(8)} = -0.656$, $P = 0.53$, Cohen's d = − 3.634) (Fig. 2, Table 3).

Gene expression

The expression levels of *AQP1* ($t_{(11)} = 2.792$, $P = 0.031$, Cohen's d = 1.493), *AQP3* ($t_{(11)} = 3.721$, $P = 0.008$, Cohen's d = 1.997), *NCC* ($t_{(11)} = 2.213$, $P = 0.049$, Cohen's d = 1.278), *nNOS* ($t_{(11)} = 2.439$, $P = 0.046$, Cohen's d = 1.309), *CHRM2* ($t_{(11)} = 2.542$, $P = 0.04$, Cohen's d = 1.364), and *ADRB2* ($t_{(11)} = 5.067$, $P = 0.002$, Cohen's d = 2.713) in the small intestine of *E. multiocellata* were higher in winter than in summer; however, *NKCC2* expression showed no significant differences between winter and summer ($t_{(11)} = 1.471$, $P = 0.169$, Cohen's d = 0.841) (Fig. 3). Moreover, the expression levels of *AQP1* ($t_{(11)} = -6.579$, $P < 0.001$, Cohen's d = − 3.727), *AQP3* ($t_{(11)} = -5.778$, $P = 0.001$, Cohen's d = − 0.3.319), and *nNOS* ($t_{(11)} = -3.388$, $P = 0.006$, Cohen's d = − 1.85) in the large intestine were lower in winter than in summer, whereas the expression levels of *NCC*

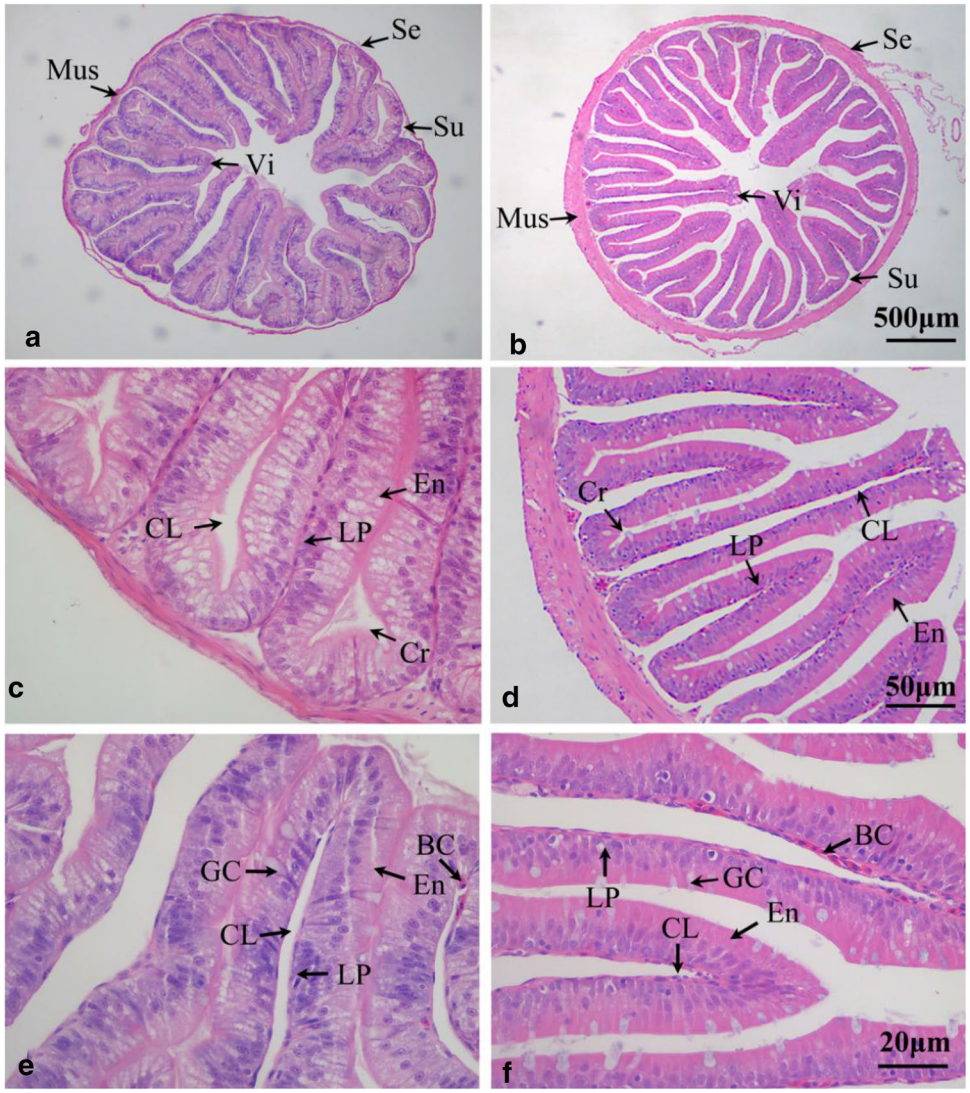

Fig. 1 Histological structure of the small intestine of *E. multiocellata* during summer and winter. **a**, **b**, the small intestines during summer and winter, scale bar = 500 μm; **c**, **d**, the inner wall of the small intestine during summer and winter, scale bar = 50 μm; **e**, **f**, the small intestinal villus during summer and winter, scale bar = 20 μm. *BC* blood capillary, *CL* central lacteal, *Cr* crypt, *En* enterocyte, *GC* goblet cells, *LP* lamina propria, *Mus* muscle, *Se* serosa, *Su* submucosa, *Vi* villus

($t_{(11)} = 5.202$, $P = 0.002$, Cohen's d = 2.782) and *CHRM2* ($t_{(11)} = 2.814$, $P = 0.025$, Cohen's d = 1.515) were higher in winter than in summer. Lastly, the expression levels of *NKCC2* ($t_{(11)} = 0.816$, $P = 0.432$, Cohen's d = 0.464) and *ADRB2* ($t_{(11)} = 0252$, $P = 0.806$, Cohen's d = 0.14) showed no significant differences between winter and summer (Fig. 3).

Discussion

In this study, the comparison of intestinal microanatomy of *E. multiocellata* demonstrated seasonal heterogeneity in its morphology. Several morphological variations, including mucosal thickness, the villus height, and the epithelial cell height, may be critical for improving nutrition absorption during hibernation season. In addition, the intestinal expression of *AQP1, AQP3, NCC, nNOS, CHRM2,* and *ADRB2* responded to seasonal changes, and they were differentially expressed in the small and large intestines of *E. multiocellata*. Overall, these results indicate that the gut of *E. multiocellata* is remarkably flexible in coping with seasonal changes and variable energy demands.

Variations in intestine mass between winter and summer

The body mass of *E. multiocellata* was lower in winter than in summer owing to the ceased food intake during hibernation, and the relative mass of the small and large intestines was greater in winter than in summer. In contrast, no significant seasonal differences were observed in the mass of the small or large intestines. One possible explanation for these results arises from a previous report which found that, in amphibians and reptiles, intestinal mass reached its maximum value immediately after the ingestion of food (i.e., between 1 and 3 days post-feeding) and then rapidly fell

Table 3 Intestinal histomorphology of *E.multiocellata* during summer and winter

Histological structures (μm)	Summer (n = 5)	Winter (n = 5)
Small intestine		
Mucous thickness	403.59 ± 15	478.23 ± 21.71*
Submucosa thickness	20.93 ± 2.93	9.04 ± 0.69*
Muscularis thickness	51.6 ± 0.99	79.35 ± 15.69
Villus width	81.54 ± 2.91	139.44 ± 12.85*
Villus height	264.32 ± 3.62	316.67 ± 9.8*
Enterocyte diameter	7.8 ± 0.27	7.98 ± 0.6
Enterocyte height	37.58 ± 0.85	48.82 ± 1.12*
Crypt depth	50.07 ± 2.14	51.94 ± 3.81
Large intestine		
Mucous thickness	524.24 ± 13.23	608.2 ± 15.94*
Submucosa thickness	20.27 ± 1.83	29.78 ± 1.04*
Muscularis thickness	211.67 ± 0.24	169.38 ± 5.25*
Enterocyte diameter	5.72 ± 0.24	5.44 ± 0.34
Enterocyte height	39.58 ± 1.92	61.84 ± 3.36*

Values are expressed as Means ± SE. Asterisk (*) indicate significant difference ($P < 0.05$)

to fasting values as digestion proceeded (Zaldúa and Naya 2014). Incidentally, in a study on the Andean toad (*Bufo spinulosus*), no differences in large intestine mass were observed between feeding, fasting, and hibernation (Naya et al. 2009a); however, in studies on the Andean lizard species *Liolaemus nigroviridis* and *L. moradoensis*, the mass of the small intestine was larger in summer than in winter (Naya et al. 2009b; 2011). Moreover, other studies have indicated that Djun garian hamsters (*Phodopus sungorus*) and the rufous-collared sparrow (*Zonotrichia capensis*) both exhibited increased intestinal mass for survival during cold winter months (Novoa et al. 1996; Piscitiello et al. 2020). Therefore, there seems to be a wide variation in the ways in which intestinal mass change in winter in different species. We do not expect species with different diets and lifestyles to respond similarly.

Variations in intestinal histomorphology between winter and summer

Tissue remodeling of the small intestine is known to help coordinate seasonal demands (Do Nascimento et al. 2016). In the present study, the small intestinal villi and large intestinal folds of *E. multiocellata* were well preserved during hibernation season, which is consistent with previous findings in Tegu lizards (*Tupinambis merianae*) (Do Nascimento et al. 2016), the greater mouse-eared bat (*Myotis myotis*) (Paksuz 2014), and the thirteen-lined ground squirrel (*Ictidomys tridecemlineatus*) (Carey 1990), all of whose intestinal tissue structure remains intact during hibernation. In this study, the mucosal thickness of the small intestine and villus height were significantly greater in winter than in summer. These results correspond with those of a previous study where house sparrows (*Passer domesticus*) showed higher duodenal mucosal thickness in winter than that in summer (Lv et al. 2014). Moreover, in the present study, the villus height and width and enterocyte height were increased in *E. multiocellata* in winter compared with those in summer. Absorption is known to occur at the apical and basolateral membranes of the villi's enterocytes, and therefore, the number, length, and morphological structure of intestinal villi influence the digestive and absorption functions of the digestive tract (Pluske et al. 1996). These changes increase the contact area between nutrients, water, and the intestine, as well as facilitate the exchange of material between cells and the external environment and promote the absorption of nutrients and water. Thus, *E. multiocellata* may increase intestinal absorption by changing mucosal histology during hibernation. Although foraging activity ceased during hibernation, there may have been a small amount of feed residues in the intestines since the hibernated group was sacrificed in mid-hibernation periods (December). Hence, a possible explanation could be an increment in some intestinal structure in winter in a compensatory manner that enables intestinal nutrient absorption.

The thickness of the submucosa was significantly higher in summer than in winter, possibly enhancing the self-buffering and protective capacity of the small intestine during this season when the lizard is heavily fed (Lv et al. 2014). However, the relatively thin submucosa during winter is sufficient to support local absorption. Notably, the muscularis externa of the gut wall, responsible for motility, is primarily composed of smooth muscle cells. However, in this study, its thickness and crypt depth in the small intestine did not show seasonal changes, indicating that muscularis thickness in the small intestine tends to remain relatively stable during hibernation, which is consistent with the findings of certain fasted anurans (Secor 2005).

The ambient temperature is independent of other environmental factors (e.g., diet quality and photoperiod) that tend to trigger the onset of responses allowing the maintenance of body condition (Del Valle et al. 2004). Studies have demonstrated that the structure of small intestinal mucosa may undergo plastic changes in cold environments. For example, in Brandt's vole (*Lasiopodomys brandtii*), cold acclimation increased the villus length and number of endothelial lymphocytes in the small intestine (Bo et al. 2018). Furthermore, in Gansu Zokor (*Myospalax cansus*), the thicknesses of the mucosa and muscular layer, as well as the height of the intestinal villus, were found to be higher in winter than in summer (Wang et al. 2016). Winter acclimatization is certain to comprise multiple complex and interacting adjustments (Heldmaier and

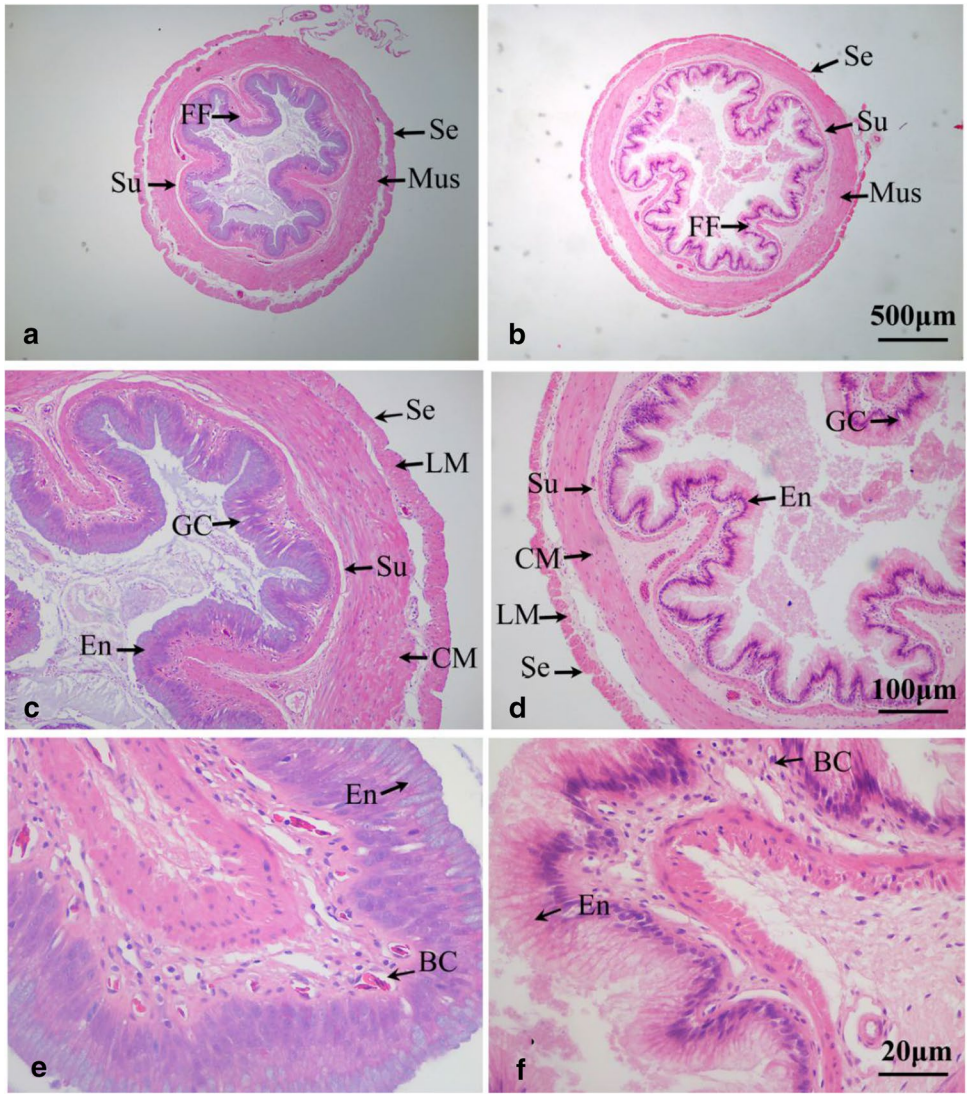

Fig. 2 Histological structure of the large intestine of *E. multiocellata* during summer and winter. **a**, **b**, the large intestines during summer and winter, scale bar = 500 μm; **c**, **d**, the inner wall of the large intestine during summer and winter, scale bar = 100 μm; **e**, **f**, the large intestinal fold fissure during summer and winter, scale bar = 20 μm. *BC* blood capillary, *CM* circular muscles, *En* enterocyte, *FF* fold fissure, *GC* goblet cells, *LM* longitudinal muscle, *Mus* muscle, *Se* serosa, *Su* submucosa

Lynch 1986). Hence, the increase of some intestinal tissue may result from the comprehensive influence of ambient temperature and fasting.

The large intestine is an important absorption site for water, electrolytes, and cellulose, hence the changes therein are primarily reflected in the regulation of water balance in animals (Rechkemmer and Engelhardt 1993). The thicknesses of the mucosal layer and submucosa, as well as the enterocyte height, were found to increase in winter, which would support the transport of water and electrolytes. The thin muscle layer of the large intestine which was observed in this study might be related to fasting during hibernation and the subsequent weakening of the large intestine's movement; therefore, regulating intestinal structure according to functional demand may be an important mechanism for energy conservation.

Variations in the intestinal water-salt regulating factors between winter and summer

AQPs are divided into several subtypes. AQP1, for example, is a strict protein that only allows the passage of water, whereas AQP3 is a channel that promotes glycerol permeability and water transport, as well as urea flow (Masyuk et al. 2002). Water absorption in the small intestine primarily occurs through a paracellular pathway. AQPs play a significant role in transcellular water transport (Masyuk et al. 2002). Here, *AQP1* and *AQP3* in the small intestine of *E. multiocellata* showed a higher expression in winter than in summer, suggesting that *AQP1* and *AQP3* may be involved in transcellular water transport in the small intestine during hibernation season.

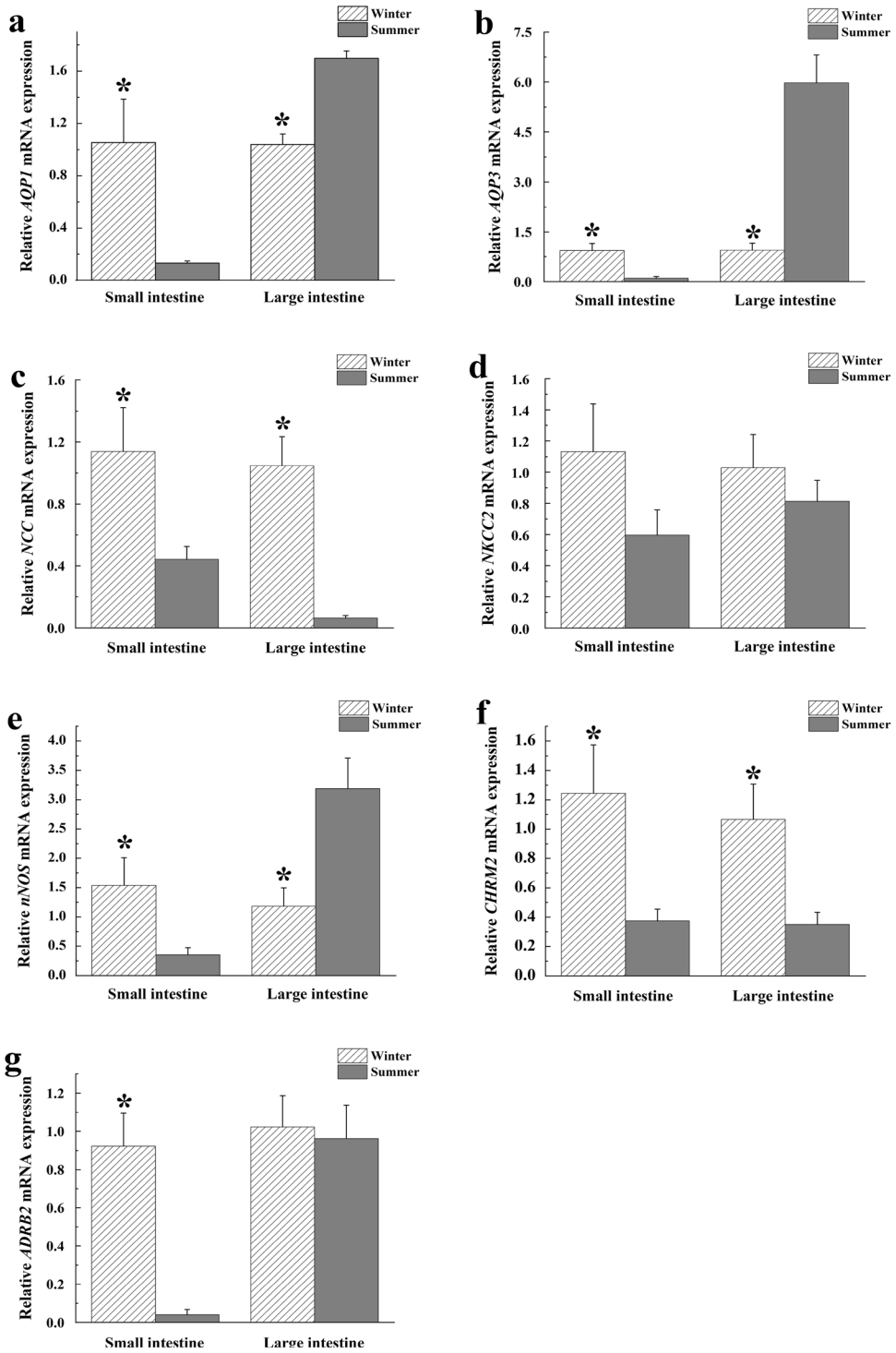

Fig.3 Intestinal expression of *AQP1*, *AQP3*, *NCC, NKCC2, nNOS*, *CHRM2*, and *ADRB2* in *E. multiocellata* during summer and winter. Values are expressed as Means ± SE. Asterisk (*) indicate significant difference ($P < 0.05$)

In the colon of mammals, water absorption is mostly transcellular and is mediated by AQPs, including AQP1 and AQP3 (Gallardo et al. 2002; Bozinovic and Gallardo 2006; Ikarashi et al. 2012; Laforenza 2012). AQP1 contributes to the passage of water between the gastrointestinal mucosa and the bloodstream (Laforenza 2012), while a decrease in AQP3 expression in the colon inhibits water absorption from the luminal to the vascular side (Ikarashi et al. 2012). In this study, compared with that in summer, *AQP1* and *AQP3* expression in the large intestine significantly decreased in winter, suggesting the attenuation of water absorption in the large intestine through *AQP1* and *AQP3* during hibernation season. This is similar to the renal expression of *AQP1* and *AQP3* in hibernating *E.*

multiocellata (Zhong and Wang 2022). The large intestine of *E. multiocellata* likely functions throughout hibernation at low levels; in contrast, during the active period (in summer), the high expression of *AQP1* and *AQP3* in the large intestine might play an important role in the complete absorption of water and glycerol from food.

Notably, *AQP1* and *AQP3* expression in the large intestine was inconsistent with that in the small intestine. One potential explanation for this result is the many physiological functions of AQPs in the gastrointestinal tract that are dependent on their distribution and localization (Laforenza 2012; Lv et al. 2014). Although both paracellular and transcellular water transport likely occur in the epithelia of the small and large intestines, their relative contributions may vary (Masyuk et al. 2002). In the small intestine, water is absorbed via isotonic mechanisms, while in the large intestine, it is absorbed against an osmotic gradient (Laforenza 2012). Moreover, AQPs are responsible for osmotically driven transmembrane water movements in the colon (Laforenza 2012). In view of the above evidence, we speculate that the regulatory performance and contributions of AQPs may differ in the water transport of small and large intestines, resulting in their differential expression in *E. multiocellata*.

The balance of ions and water is a critical challenge for desert reptiles (Vistro et al. 2019), and the osmoregulatory function of the small intestine is the most common strategy of the body to maintain this balance in the face of various environmental changes (Hu et al. 2013). Decreased water intake causes an increase in plasma sodium and osmotic pressure; therefore, hibernating animals have high plasma sodium concentrations and osmotic pressure (Jani et al. 2013). For example, plasma sodium and chloride levels in soft-shelled turtles (*Pelodiscus sinensis*) significantly increase during hibernation compared to the non-hibernation period (Vistro et al. 2019). NCC and NKCC2 are known to support passive water transport and ion absorption in the intestine (King et al. 2004; Hamann et al. 2005). In this study, the expression of *NCC* in both small and large intestines was higher in winter than in summer, indicating that the *NCC* gene could be activated in *E. multiocellata* during hibernation. Further, NCC plays an important role in intestinal osmoregulation, and it is necessary to provide an osmotic gradient for water and minerals transport in *E. multiocellata*. NKCC2 mRNA and protein expression levels were reportedly enhanced in the small intestine of soft-shelled turtles during hibernation compared to the non-hibernation period (Vistro et al. 2019). In this study, although there was no seasonal difference in intestinal *NKCC2* expression in *E. multiocellata*, there appeared to be an upmodulated trend in winter, indicating that *NKCC2* together with *NCC* may facilitate water-salt balance during hibernation.

Variations in the intestinal motility regulating factors between winter and summer

Neunlist and Schemann (2014) proposed that the presence or absence of nutrients in the intestinal lumen induces long-term changes in neurotransmitter expression, excitability, and neuronal survival, ultimately affecting intestinal motility, secretion and permeability. To overcome the challenges of harsh environmental conditions, effective absorption and digestion depend not only on structural regulation but also on neural control mechanisms. In this study, a high expression of *nNOS, CHRM2,* and *ADRB2* in the small intestine of *E. multiocellata* was found during winter. The gastrointestinal motility accelerates when the amount of NO decreases (Stark and Szurszewski 1992); nNOS slows peristalsis in the small intestine and allows the full absorption of water and nutrients (Nase and Boegehold 1997; Taksande et al. 2011); *ADRB2* is involved in the relaxation of intestinal smooth muscle (Kamiar et al. 2021) and slows down peristalsis in the small intestine. CHRM2 increases contraction amplitude, tension, and peristalsis and promotes gastric and intestinal secretions (Stengel et al. 2000; Jeong et al. 2017). Here, the high expression of *nNOS* and *ADRB2* during hibernation suggests that NO and ADRB2 synthesis or release increases, slowing down peristalsis in the small intestine. However, a high expression of *CHRM2* would promote intestinal peristalsis. Its role seems to be inconsistent with *nNOS* and *ADRB2*, which may be owing to the following reasons. The enteric reflex circuitry regulates motility through both excitatory and inhibitory neural outputs to smooth muscle cells (Mazet 2014). ACh and NO, as the main excitatory and inhibitory neurotransmitters of the gastrointestinal smooth muscle, respectively, do not strictly operate in accordance with the classical view of enteric neuromuscular transmission (Mazet 2014). Hence, there may be a delicate balance between cholinergic and nitroergic neurotransmitters, in which they jointly regulate intestinal movement. Alternatively, owing to the neurochemical properties (e.g., co-expression of distinct transmitters, receptors, and ion channels) of enteric neurons (Holzer et al. 2001), although the expression of these genes in the gut was up- or downregulated in this study, we could not exclude an integrated effect of these regulatory factors on intestinal motility.

The expression of *nNOS* in the large intestine of *E. multiocellata* during summer was significantly low, which may help promote defecation. During hibernation, high levels of *CHRM2* expression were observed in the large intestine; however, no significant changes were observed in the expression of *ADRB2* in the large intestine, suggesting that *ADRB2* expression was relatively stable in the large intestine throughout both winter and summer. The gastrointestinal tract has complex motor pattern and secretory activities, while peristaltic regulation exhibits spatiotemporal characteristics (Holzer et al.

2001). The specific functions of the small and large intestines are different, and the nNOS, ADRB2, and CHRM2 may be differentially distributed throughout the small and large intestine, which may determine the discrepancies in modulation and expression of the aforementioned genes. Finally, the degree of gut flexibility depends on the complex interaction between taxa and nutrients (Karasov et al. 2011). As digestion in reptiles is considerably slower than that in most mammals owing to the differences in meal frequency (Secor et al. 1994), the gut motility adjustment of *E. multiocellata* may be particular, that is, different from that of mammals.

In conclusion, this study found that *E. multiocellata* responds to hibernation season by altering its intestinal histological features and gene expression. In winter, *AQP1, AQP3, NCC, nNOS, CHRM2,* and *ADRB2* were upregulated in the small intestine, and *NCC* and *CHRM2* were upregulated in the large intestine, with downregulation of *AQP1*, *AQP3*, and *nNOS*. These results indicate that intestinal water-salt transport activity is flexible in terms of seasonal changes, in that AQPs together with $Na^{(+)}$ transporters play an important mediating role in transport capacity, while *nNOS, CHRM2,* and *ADRB2* regulate intestinal motility; the physiological flexibility of the small and large intestines may be discrepant due to their functional differences. These findings suggest that the phenotypic flexibility of the gut is necessary to allow hibernating reptiles to successfully overcome environmental challenges in winter. However, as hibernation implies different adjustments at the genetic, molecular, biochemical, tissue, and cellular levels, further integrative studies are needed to assess seasonal flexibility.

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Data availability The data sets generated during and (or) analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare no competing or financial interests.

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