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Ups and downs of intestinal function with prolonged fasting during aestivation in the burrowing frog, *Cyclorana alboguttata*

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SUMMARY

Although green striped burrowing frogs (*Cyclorana alboguttata*) experience large reductions in the mass and absorptive surface area of the small intestine (SI) during aestivation, little is known about how this may affect the functional capacity of the SI. We examined changes in the function (L-proline uptake rate and capacity) and metabolism of the SI (*in vitro* oxygen consumption, Na^+/K^+-ATPase activity and abundance) of *C. alboguttata* following 6 months of aestivation. L-Proline uptake rate was significantly higher in aestivating frogs, but overall uptake capacity was lower than in active frogs. Total SI oxygen consumption rate ($\dot{V}_{O_2}$) was also lower in aestivating frogs, despite no difference in mass-specific $\dot{V}_{O_2}$. The proportion of intestinal $\dot{V}_{O_2}$ associated with Na^+/K^+-ATPase activity and protein synthesis was equivalent between active and aestivating frogs, suggesting these processes were unaffected by aestivation. Indeed, the activity of Na^+/K^+-ATPase transporters in the SI of aestivating frogs was not different from that of active animals. Aestivating frogs maintained Na^+/K^+-ATPase activity, despite experiencing a reduction in the density of Na^+/K^+-ATPase transporters, by increasing the molecular activity of the remaining pumps to 2–3 times that of active frogs. These results show that functionality of the SI is maintained at the cellular level, potentially facilitating the reclamation of nutrients from the intestinal lumen while in aestivation. Despite this, the functional capacity of the SI in aestivating *C. alboguttata* is significantly reduced due to a reduction in tissue mass, helping frogs to conserve energy while in aestivation.

Key words: aestivation, small intestine, fasting, *Cyclorana alboguttata*, Na^+/K^+-ATPase activity, protein synthesis, L-proline uptake.

INTRODUCTION

The functional small intestine (SI) has a disproportionately large energetic demand relative to other organs (Cant et al., 1996) and consequently, for those animals that experience prolonged episodes of food deprivation, there may be a selective pressure to reduce energy expenditure by the intestine during fasting. In almost all cases, fasting results in a reduction in SI mass and surface area which serves to reduce energy expenditure by the organ (Biebach et al., 1986; Carey, 1990; Secor et al., 1994; Tracy and Diamond, 2005; Starck et al., 2007). These morphological responses to fasting often result in concomitant changes to the functional capacity (i.e. the ability to digest and assimilate ingested nutrients) of the tissue which in turn may compromise the ability of the animal to restore digestive efficiency upon re-feeding (for review, see Ferraris and Carey, 2000).

Many species of amphibians are capable of withstanding extended periods of unfavourable environmental conditions, condensing their lives into brief periods of activity when conditions allow (Pinder et al., 1992). The green striped burrowing frog *Cyclorana alboguttata* may spend upwards of 10 months of the year underground in a hypometabolic state of aestivation (E.A.M., personal observation). Previously we have shown that fasting during aestivation has a considerable impact on the morphology of the SI of *C. alboguttata* (Cramp and Franklin, 2003; Cramp et al., 2005). During aestivation (3–9 months), the mass, length and absorptive surface area of the SI decline significantly (by up to 80%) (Cramp et al., 2005). We hypothesize that these morphological changes will reduce the ability of the SI to assimilate nutrients during aestivation but will represent marked energy savings to the animal and assist in conserving endogenous energy reserves.

In the present study we sought to examine indices of SI function which may affect nutrient assimilation capacity during aestivation in *C. alboguttata*. To this end we measured uptake of the amino acid L-proline by the SI of active *C. alboguttata* and after 6 months of aestivation. As complex membrane proteins, intestinal nutrient transporters are inherently expensive to produce and maintain (Cant et al., 1996). Membrane protein transporters are synthesized from intracellular amino acid pools at a significant energy cost (McBride and Kelly, 1990), while their activity requires the constant maintenance of a membrane potential facilitated by the activity of energetically expensive Na^+/K^+-ATPase pumps on the basolateral membrane of enterocytes (Ferraris and Carey, 2000). Given the significant cost of production and activity of nutrient transporters, it is not surprising that many amino acid transporters are tightly regulated (Karasov and Diamond, 1983a). The plasticity of intestinal nutrient transporter production and activity enables the intestine to 'tailor' energy expenditure to the digestive state of the organism by reducing or increasing nutrient uptake capacity. An amino acid was selected as the substrate of choice in this study since adult *C. alboguttata* are carnivorous and therefore consume primarily protein (amino acids) in their diet. L-Proline uptake has also been used to assess the functional state of the SI in similar studies on hibernating ground squirrels (Carey, 1990; Carey and Sills, 1992), fasting snakes (Diamond and Karasov, 1987; Secor et al., 1994; Secor and Diamond, 1995) and fasting amphibians (Secor, 2005).

To examine the energy costs associated with the regulation of nutrient transport rates, we examined the *in vitro* metabolic rate ($\dot{V}_{O_2}$) of the SI and the relative contributions of Na^+/K^+-ATPase activity and protein synthesis to the $\dot{V}_{O_2}$ of the tissue. These two cellular processes underpin many of the absorptive activities of the intestine

and may account for between 50% and 90% of the total oxygen consumed by the gastrointestinal tract of some animals (McBride and Kelly, 1990). Changes in the rates of these processes can markedly affect the rate of energy expenditure by the tissue and in turn alter its functional (absorptive) efficiency (McNurlan et al., 1979; McBride and Milligan, 1985b; Huntington and McBride, 1988; Cant et al., 1996). An increase or decrease in the activity of the Na^+/K^+-ATPase pump respectively facilitates or reduces the absorption of nutrients by the SI (Kelly and McBride, 1990). Therefore, we quantified the activity, abundance and relative molecular turnover rates of Na^+/K^+-ATPase transporters in the SI of active and aestivating frogs to determine whether changes in nutrient transport rates correlate with changes in the underlying properties of the Na^+/K^+-ATPase transporters of the SI.

MATERIALS AND METHODS

Experimental animals

Cyclorana alboguttata Günther 1867 were collected from flooded roadsides at numerous sites throughout Queensland, Australia. Animals were transported to The University of Queensland and housed individually in 5 l plastic containers lined with moist paper towels. Frogs were allowed 1 week to recover from transportation before experimentation. All animals were maintained at 24±2°C with a 12 h:12 h light:dark regime. To induce aestivation, animals were placed into 5 l plastic containers containing clay soil (from their original habitats) saturated with water. Having burrowed into the mud, animals were left undisturbed in aestivation for approximately 6 months. Active (control) animals were fed once a week (~5% body mass) on live cockroaches and crickets, and were killed 36–48 h post-feeding. Aestivating animals were killed while aestivating. All procedures were undertaken with the approval of The University of Queensland Animal Welfare committee and the Queensland Environmental Protection Agency.

Intestinal L-proline uptake

Nutrient transport capacity by the SI was determined using the everted sleeve protocol described by Karasov and Diamond (Karasov and Diamond, 1983b). Previous work by Secor (Secor, 2005) has demonstrated that this technique does not damage the intestine of fed and fasted frogs. Animals (N=6 per group) were killed by double pithing and the middle region of the SI removed and sectioned into 1 cm sleeves. The SI was differentiated from the colon by a marked increase in diameter of the intestine upon transition to the colon. Sleeves were everted and secured to a glass rod. Adjacent segments were used to separate Na^+-dependent L-proline uptake from diffusional (Na^+-independent) uptake. One segment from each pair was preincubated in Na^+-free Ringer solution (containing, in mmol l^{-1}: 83.8 choline chloride, 4 KCl, 1.8 $CaCl_2$, 0.8 $MgSO_4$, 0.8 KH_2PO_4, 17.8 choline HCO_3 and 11 glucose) and one in normal Ringer solution (containing, in mmol l^{-1}: 83.8 NaCl, 4 KCl, 1.8 $CaCl_2$, 0.8 $MgSO_4$, 0.8 NaH_2PO_4, 17.8 $NaHCO_3$ and 11 glucose) both at 23°C for 5–10 min. Following preincubation, sleeves were incubated in Ringer solution (Na^+ and Na^+-free) containing 25 mmol l^{-1} L-proline (isosmotically replacing sodium chloride or choline chloride) and 2 μCi ml^{-1} of L-[2,6-^{3}H]proline (Amersham, Sydney, Australia) together with an adherent fluid marker, 0.1 μC ml^{-1} ^{14}C polyethylene glycol (PEG) (Amersham). Following incubation, tissues were removed from rods, lightly blotted, weighed, placed into 20 ml glass scintillation vials and solubilized in 1 ml of Soluene-350 Tissue Solubilizer (Perkin Elmer, Melbourne, Australia) for 2 h at 60°C. Ultima-Gold Liquid Scintillation Cocktail (10 ml; Perkin Elmer) was then added to vials and samples were loaded into a United Technologies Packard Tri-Carb 4000 Liquid Scintillation System and counted for 3×5 min each. The mean number of disintegrations per minute was recorded for both isotopes. L-proline uptake rate is presented as nmol L-proline min^{-1} mg^{-1} wet tissue mass. Total L-proline uptake capacity was estimated by extrapolating maximal L-proline uptake min^{-1} mg^{-1} wet tissue mass over the entire mass of SI. Na^+-dependent transport was calculated by subtracting the rate of uptake of L-proline under Na^+-free conditions from the net rate of uptake of L-proline (Na^+-present conditions). L-Proline uptake rates were also normalized to DNA content using data from subsequent experiments (see below).

In vitro intestinal oxygen consumption

The *in vitro* oxygen consumption rate ($\dot{V}_{O2}$) of the SI was measured as per Fuery et al. (Fuery et al., 1998). The proportion of intestinal $\dot{V}_{O2}$ associated with Na^+/K^+-ATPase activity or protein synthesis was also determined, using ouabain and cycloheximide, respectively, to inhibit these processes. Active (N=7) and aestivating (N=7) animals were killed and the SI removed. The intestine was gently rinsed with cold (4°C) amphibian Ringer solution (containing, in mmol l^{-1}: 111 NaCl, 2.5 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 5 Hepes and 10 glucose) bubbled with air, blotted dry and weighed. The SI was opened along the mesenteric border and sectioned transversely into 1 mm wide pieces. Two to four pieces of intestine were then placed into each of three treatments at 23°C: 10 ml of fully aerated Ringer solution containing 1 mmol l^{-1} ouabain (Na^+/K^+-ATPase inhibitor), 0.1 mmol l^{-1} cycloheximide (protein synthesis inhibitor) or Ringer solution only (control treatment). Tissue pieces were pre-incubated for 15 min on an orbital shaker before being transferred into individual 3 ml plastic syringes containing 2 ml of fully re-aerated Ringer solution from the preincubation vessel. Measurements of initial oxygen concentration were made by injecting 1 ml of Ringer solution from each syringe through a MC100 Microcell containing a 1302 oxygen electrode (Strathkelvin Instruments, Motherwell, UK). The electrode was connected to a Strathkelvin 782 dual channel oxygen meter. The electrode and meter were calibrated with air-saturated distilled water and saturated sodium thiosulphate (zero) solution. The syringes were then sealed with a 3-way stopcock, placed on a rocking platform, covered with aluminium foil and left undisturbed for 90 min. Blanks (duplicate syringes containing Ringer solution only) were also sealed and left for the 90 min incubation period to correct for any background changes in oxygen concentration. In a related but as yet unpublished study (R.L.C. and C.E.F.), we determined that the *in vitro* metabolic rate of the SI declined most rapidly approximately 2 h after removal from the animal. Prior to this, the decline of intestinal metabolic rate was relatively stable. Hence, all metabolic rate measurements were taken within 2 h of removal from the animal. At the completion of the incubation period, the oxygen concentration of the syringes was measured as described above. The tissue pieces were then removed from the syringes, blotted dry and weighed. Tissue pieces were frozen at –20°C for subsequent DNA quantification.

The $\dot{V}_{O2}$ for each tissue piece was determined using the following equation:

$$\dot{V}_{O_2} = \frac{(V \times (O_{2,i} - O_{2,f}))/t}{m} \quad ,$$

where V is volume of Ringer solution (l), m is the mass of the tissue slice (g), $O_{2,i}$ and $O_{2,f}$ are the initial and final concentrations of oxygen in the Ringer solution, respectively, and t is the period during which the chamber was sealed (h). Rates of oxygen consumption

were also normalized to nucleic acid content after determination of DNA content (see below).

Total intestinal $\dot{V}_{O_2}$ was estimated by extrapolating from the $\dot{V}_{O_2}$ of intestinal pieces incubated in Ringer solution only (control treatment). The proportion of intestinal oxygen consumption associated with Na^+/K^+-ATPase activity and protein synthesis was determined by subtracting the $\dot{V}_{O_2}$ of tissue pieces incubated in ouabain and cycloheximide, respectively, from that in the control treatment.

Na^+/K^+-ATPase activity

Na^+/K^+-ATPase activity (N=6 active, N=6 aestivating frogs) was determined using the protocol described by Else (Else, 1994). Intestinal tissues were collected from animals as described and immediately snap-frozen in liquid nitrogen, and stored at –80°C until assaying. Tissue homogenates (10%, w/v) were prepared in ice-cold homogenization buffer (in mmol l^{-1}: 250 sucrose, 5 EDTA, 20 imidazole and 2.4 sodium deoxycholate at pH 7.0). Homogenates (50–100 µl) were preincubated in assay medium [in mmol l^{-1}: 83.3 Tris (hydroxymethyl) aminomethane HCl, 5 $MgCl_2$, 100 NaCl, 15 KCl and 5 NaN_3, pH 7.5] for 10 min at room temperature either with or without ouabain (1 mmol l^{-1}). Na^+/K^+-ATPase activity was determined as the difference in phosphate (P_i) liberation between the two incubations. The reaction was started by the addition of Na_2-ATP (10 mmol l^{-1}) and continued for 10 min. The reaction was stopped by the addition of an equal volume of ice-cold perchloric acid (0.8 mol l^{-1}). The reaction mixture was centrifuged at 1200 ***g*** for 20 min at 0–4°C. Phosphate concentration was measured using the PiBlue™ phosphate assay kit (BioAssay Systems, Hayward, CA, USA). Protein concentration of homogenates was measured using the Bradford method. All assays were performed in duplicate or triplicate. Na^+/K^+-ATPase activity is expressed as pmol P_i mg^{-1} wet tissue mass h^{-1}, pmol P_i mg^{-1} protein h^{-1} and pmol P_i mg^{-1} DNA h^{-1}.

Density of Na^+/K^+-ATPase pumps

To determine the density of functional Na^+/K^+-ATPase pumps, we used a ouabain binding protocol as described by Else (Else, 1994). The SI was removed from animals as described (N=6 per treatment group), cut into 2–20 mg pieces and placed into 10 ml ice cold oxygenated (95% O_2, 5% CO_2) K^+-free Ringer solution (containing, in mmol l^{-1}: 115 NaCl, 20 $NaHCO_3$, 3.1 NaH_2PO_4, 1.4 $MgSO_4$, 1.3 $CaCl_2$ and 16.7 glucose; pH 7.4). Tissues samples were incubated in K^+-free Ringer solution for 2×10 min on ice. Individual tissue pieces were placed into K^+-free Ringer solution at room temperature containing 1 µCi [^{3}H]ouabain ml^{-1} (GE Healthcare Bio-Sciences, Rydalmere, NSW, Australia) plus additional 'cold' ouabain to make up final concentrations ranging from 0.2 to 50 µmol l^{-1}. An additional concentration of 10^{-3} mol l^{-1} ouabain was used to correct for non-specific binding. Tissue pieces were incubated for 2 h at room temperature, and were gassed continuously with carbogen. At the conclusion of the incubation, tissue pieces were washed with ice-cold K^+-free medium (5×8 min), blotted lightly and weighed. Tissues were then solubilized in 1 ml Solvable (Perkin Elmer Victoria, Australia) at 60°C for 2–4 h, allowed to cool, then 10 ml of UltimaGold scintillation fluid (Perkin Elmer) was added and samples were counted (d.p.m.).

Ouabain binding was expressed per unit tissue wet mass or per mg DNA. Specific binding was calculated by subtracting the total activity measured in excess ouabain (10^{-3} mol l^{-1}), which was considered to be non-specific binding, from all other measurements. [^{3}H]Ouabain binding sites per g tissue or per µg DNA were determined by multiplying the specific uptake by the total ouabain concentration in the medium. Non-linear regression analysis was used to determine maximum specific binding (i.e. sodium pump density; B_{max}) and was calculated assuming a 1:1 stoichiometry between sodium pump units and ouabain binding sites (Turner et al., 2005). B_{max} is expressed as pmol Na^+/K^+-ATPase g^{-1} wet mass or mg^{-1} DNA.

Na^+/K^+-ATPase molecular activity

Molecular activity was defined as the rate of substrate turnover by a protein, and for the sodium pump was derived by dividing average maximal Na^+/K^+-ATPase activity per group (expressed as pmol P_i mg^{-1} wet mass min^{-1}) by the average number of sodium pumps (B_{max}, in pmol g^{-1} wet mass) per group (Else et al., 1996). The result was expressed as the number of ATP molecules hydrolysed by each sodium pump per min. Because of the small amounts of tissue available, particularly in aestivating animals, Na^+/K^+-ATPase activity and ouabain binding experiments could not be performed simultaneously on tissues from the same animal. Consequently, these are from different animals and hence reported molecular activities represent estimates based on average group data from two different sets of animals. No statistical analyses were performed, nor are error bars presented.

Measurement of nucleic acid content

Total genomic DNA was extracted from intestinal tissues using a commercially available genomic DNA extraction kit (PureLink™ genomic DNA mini kit, Invitrogen, Sydney, Australia). DNA concentration was determined fluorometrically using a Qubit assay (Invitrogen).

Experimental reagents

All chemicals used were sourced from Sigma Aldrich (Sydney, Australia) unless otherwise indicated and were of laboratory grade or higher.

Statistical analyses

Student's t-tests were used to compare results between active and aestivating animals for all experiments. Analysis of the effects of ouabain and cycloheximide on intestine tissue metabolic rates was performed using a 2-way repeated measures ANOVA. Non-linear regression analysis was used to determine maximum specific ouabain binding (i.e. sodium pump density). All statistical analysis was performed using GraphPad Prism™ and all data are presented as means ± s.e.m.

RESULTS

Animal masses and SI masses

Animal masses and snout to vent lengths were not significantly different between active and aestivating treatment groups in any experiment (Table 1). SI decreased significantly (70–80%) following 6 months of aestivation (Table 1).

L-Proline uptake rate

Mass-specific L-proline uptake rate (passive plus carrier-mediated transport) was significantly (P=0.0006, t=4.967) higher in the tissues of aestivating frogs (3.395±0.3719 nmol mg^{-1} min^{-1}) compared with active frogs (1.519±0.06608 nmol mg^{-1} min^{-1}; Fig. 1A). When normalized to nucleic acid content, uptake rates were equivalent between the two treatment groups (P=0.1299; Fig. 1C). Total L-proline uptake capacity was significantly lower in aestivating frogs (0.244±0.028 µmol min^{-1}) when compared with active animals

Table 1. Animal masses, SVL and small intestinal masses from active and aestivating treatment groups

Experiment	Body mass (g)			SVL (mm)			SI mass (g)		
	Active	Aestivating	*P*	Active	Aestivating	*P*	Active	Aestivating	*P*
Small intestine MR (*N*=7 each)	17.83±1.19	21.17±1.22	n.s.	52.80±1.4	55.42±1.46	n.s.	0.27±0.04	0.06±0.01	<0.001
L-Proline uptake (*N*=6 each)	27.87±3.52	21.47±1.32	n.s.	59.73±1.24	56.46±0.69	n.s.	0.33±0.02	0.07±0.01	<0.001
Na^+/K^+-ATPase density (*N*=6 each)	28.45 ±2 .13	22.80±2.77	n.s.	61.53±0.11	58.6±0.3	n.s.	NA	NA	NA

Data are presented as means ± s.e.m. n.s., data not significantly different (*P*>0.05); NA, data not available.
MR, metabolic rate; SI, small intestine; SVL, snout to vent length.

(0.500±0.039 μmol min^{-1}; *P*=0.0004; Fig. 1B). Na^+-dependent L-proline uptake was significantly lower than Na^+-independent uptake in both active and aestivating animals (active *P*=0.047, aestivating *P*=0.0005; Fig. 1D). In this study, Na^+-dependent transport accounted for only a relatively small proportion of total L-proline uptake (13–30%). Na^+-dependent transport was not different between the two treatment groups. Na^+-independent uptake was, however, significantly greater in the SI from aestivating frogs (rank sum test, *P*=0.0022; Fig. 1D).

Intestinal oxygen consumption rate

The mass-specific metabolic rate of intestinal segments taken from active animals (114.26±6.57 μl O_2 g^{-1} wet tissue mass h^{-1}) was not significantly different from that of aestivating animals (117.93±5.5 μl O_2 g^{-1} wet tissue mass h^{-1}; *P*=0.8321, *t*=0.2167; Fig. 2A). When normalized to intestinal DNA content, however, intestinal $\dot{V}_{O_2}$ was almost 50% greater in active animals (96.95±8.22 μl O_2 g^{-1} DNA h^{-1}) than in aestivating frogs (48.91±2.32 μl O_2 g^{-1} DNA h^{-1}; *P*=0.0001, *t*=5.545; Fig. 2B). Total intestinal metabolic rate, a product of mass-specific metabolic rate and SI mass, was over 70% higher in active animals compared with aestivators (*P*<0.0001, *t*=6.533; Fig. 2C).

The rates of SI oxygen consumption (normalized to nucleic acid content) associated with both Na^+/K^+-ATPase activity and protein synthesis were not significantly different between active and aestivating frogs (Na^+/K^+-ATPase: *P*=0.8053, *t*=0.2525; protein synthesis: *P*=0.0868, *t*=1.88; Fig. 2D). 'Other' metabolic processes accounted for almost 3 times more oxygen consumption in active animals than in aestivating ones (*P*=0.0042, *t*=3.59; Fig. 2D).

Na^+/K^+-ATPase activity, sodium pump density and molecular activity

Mass-specific Na^+/K^+-ATPase activity in the SI of aestivating frogs (0.088±0.010 pmol P_i mg^{-1} tissue wet mass h^{-1}) was not significantly different from that of active frogs (0.109±0.016 pmol P_i mg^{-1} tissue wet mass h^{-1}; *P*=0.3939, *t*=1.086; Fig. 3A). Na^+/K^+-ATPase activity normalized to DNA content was also not significantly different between the two groups (*P*=0.3892, *t*=0.9001; Fig. 3B). Na^+/K^+-ATPase activity normalized to protein concentration, however, was 2.5 times higher in the SI of aestivating frogs relative to active frogs (*P*=0.001, *t*=4.594; Fig. 3C).

Na^+/K^+-ATPase transporter density (B_{max}) was significantly greater in active animals (1581±161 pmol g^{-1}) compared with aestivating animals (2360±320 pmol g^{-1}) (*P*=0.037, *F*=4.52; Fig. 4A). Similarly, Na^+/K^+-ATPase density normalized to DNA content was also greater in active animals compared with aestivating animals (4954±671 pmol μg^{-1} DNA and 1398±156 pmol μg^{-1} DNA, respectively; *P*=0.0069, *F*=7.74; Fig. 4B).

The molecular activity of the Na^+/K^+-ATPase pumps from the SI of active animals derived from mass-specific data was approximately 2 times lower than that of aestivating animals (Fig. 4C). Similarly, the molecular activity of the Na^+/K^+-ATPase

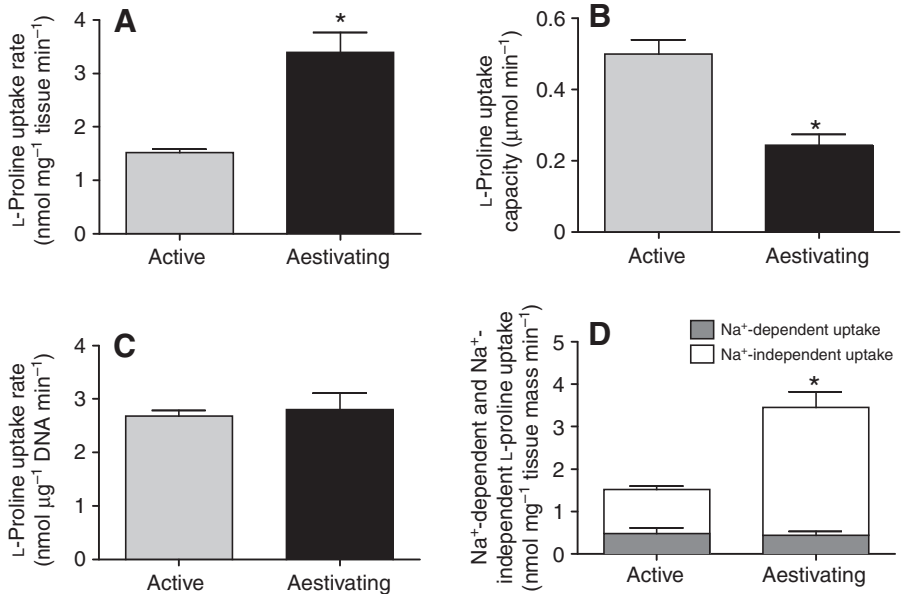

Fig. 1. The effects of aestivation on L-proline uptake by the small intestine of *Cyclorana alboguttata*. (A) Mass-specific L-proline uptake, (B) total intestinal capacity for L-proline uptake, (C) L-proline uptake rate normalized to DNA content and (D) L-proline uptake occurring *via* Na^+-dependent pathways (carrier mediated) and by Na^+-independent pathways (passive) normalized to tissue mass (mg). Aestivation resulted in a significant increase in mass-specific L-proline uptake, but a significant decrease in L-proline uptake capacity. There was no effect of aestivation on the rate of Na^+-dependent L-proline uptake, but there was a marked increase in uptake occurring *via* Na^+-independent pathways. Results are presented as means ± s.e.m. Asterisks denote a significant difference from the active group at *P*<0.05.

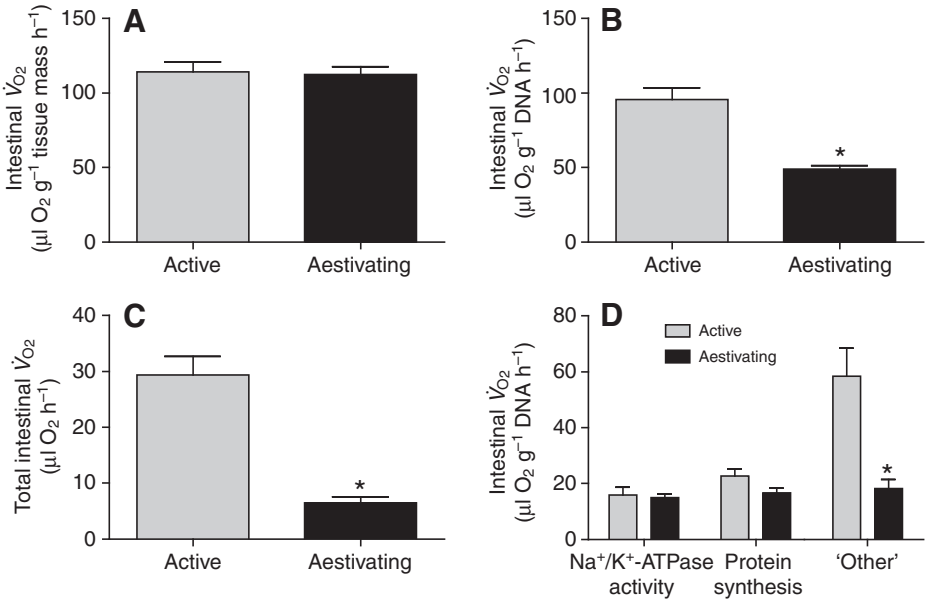

Fig. 2. The effects of aestivation on *in vitro* oxygen consumption rates ($\dot{V}_{O_2}$) of intestinal segments normalized to tissue mass (A), nucleic acid content (B) and total intestinal oxygen consumption rate (C). There was a significant reduction in total oxygen consumption rate and a 50% decline in cellular oxygen consumption rate. (D) Relative intestinal oxygen consumption rate associated with Na^+/K^+-ATPase activity, protein synthesis and 'other' metabolic processes normalized to DNA content. The amount of oxygen used to facilitate Na^+/K^+-ATPase activity and protein synthesis activity was not significantly affected by aestivation. A reduction in energy expenditure related to 'other' processes was responsible for the marked reduction in cellular oxygen consumption. Results are presented as means ± s.e.m. Asterisks denote a significant difference from the active group at $P<0.05$.

pump calculated from Na^+/K^+-ATPase activity and B_{max} data normalized to nucleic acid concentration, was approximately 3 times lower than those of aestivating animals (Fig. 4D).

DISCUSSION

Intestinal function during aestivation

In the present study, the rate of L-proline uptake by the SI was measured to assess whether intestinal absorptive function was affected by the morphological changes to the SI which occur during aestivation in the frog *C. alboguttata*. L-Proline uptake rate normalized to tissue mass was markedly greater in aestivating frogs relative to active ones. While L-proline deficient diets and fasting often result in decreased L-proline absorption rates (Diamond and Karasov, 1987; Karasov et al., 1987; Ferraris and Diamond, 1989; Secor et al., 1994), maintenance of or an increase in L-proline uptake rate during fasting has been reported in other species including hibernating ground squirrels (Carey and Sills, 1992).

Enhanced mass-specific nutrient uptake rates following prolonged food deprivation in aestivating *C. alboguttata* and hibernating ground squirrels may reflect an evolutionary strategy to alleviate the impact of profound mucosal atrophy on digestive efficiency. However, given that prolonged fasting during aestivation does result in a significant reduction in enterocyte size and number (Cramp and Franklin, 2003; Cramp et al., 2005), elevated mass-specific uptake rates in aestivating *C. alboguttata* may also reflect an increase in the number of enterocytes per unit of tissue mass. We found that the SI of aestivating frogs had 54% more DNA per unit mass suggesting a higher density of enterocytes per unit of tissue mass. Normalizing uptake rates to DNA content allows for comparisons of intestinal transport on the basis of equivalent numbers of intestinal cells. In aestivating frogs, intestinal L-proline uptake rate per unit of DNA was equivalent to that of active frogs indicating that enterocytes in the intestine of *C. alboguttata* maintain cellular L-proline uptake rates into and through aestivation. This finding demonstrates the importance of taking into account the morphological changes which occur in the SI during food deprivation in animals when assessing its function. Protecting L-proline uptake rates at the cellular level may allow aestivating *C. alboguttata* to reclaim nutrients sloughed or secreted into the intestinal lumen and might also help to ameliorate the effect of reduced absorptive surface area on nutrient assimilation capacity in re-feeding animals.

Despite the fact that the mass-specific rate of L-proline uptake was significantly higher in aestivating frogs compared with active ones, animals lose over 70% of the mass of the SI during aestivation, resulting in a reduction (50%) in the total capacity for L-proline uptake. This suggests that during aestivation in *C. alboguttata*, protecting endogenous energy stores by reducing SI mass is paramount to long-term survival and that a reduction in intestinal L-proline uptake capacity is a necessary trade-off. A loss of nutrient uptake capacity is an often reported consequence of fasting and the associated loss of SI mass and absorptive surface area (Secor and

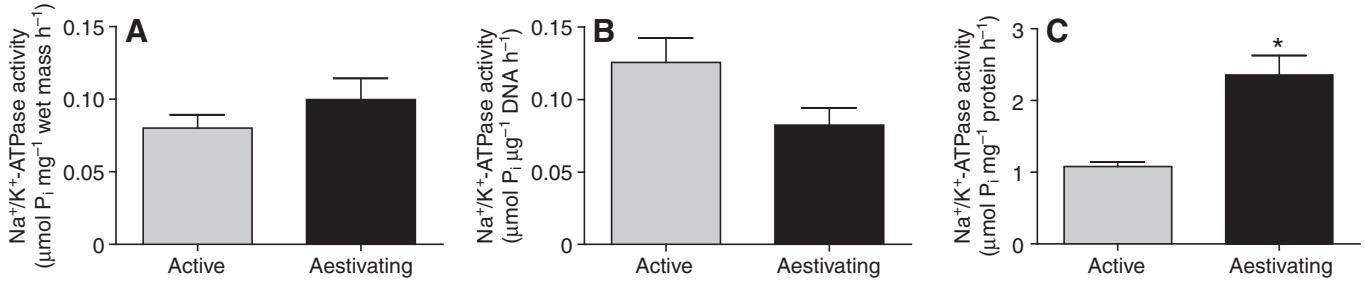

Fig. 3. The effect of aestivation on Na^+/K^+-ATPase enzyme activity in the intestine of *C. alboguttata*. (A–C) Intestinal Na^+/K^+-ATPase activity normalized to tissue mass (A), nucleic acid content (B) and protein content (C). Results are presented as means ± s.e.m. Asterisks denote a significant difference from the active group at $P<0.05$.

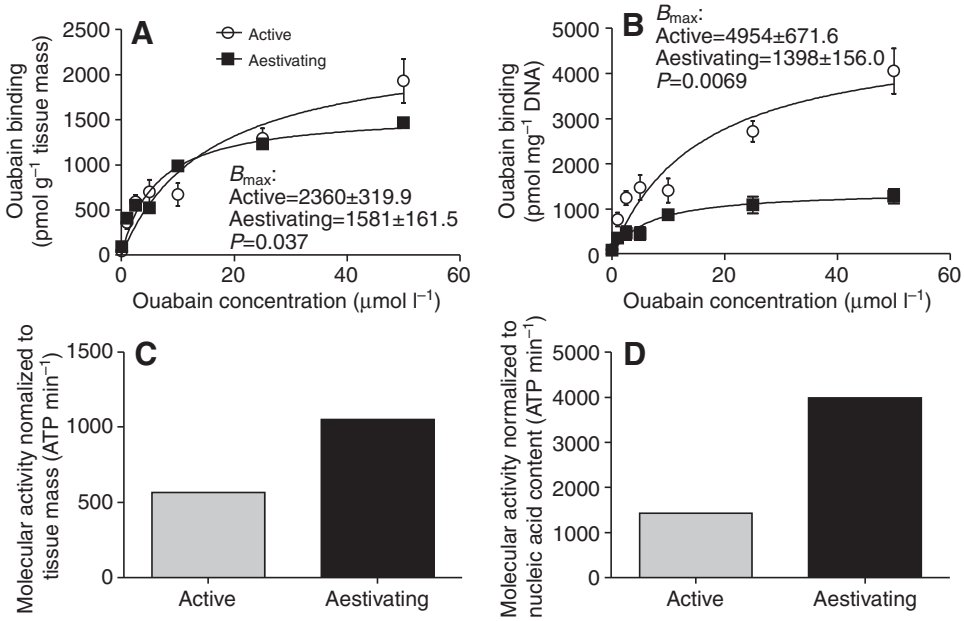

Fig. 4. The effects of aestivation on the density and molecular activity of functional Na^+/K^+-ATPase transporters in the intestinal epithelium of active and aestivating *C. alboguttata*. Density of Na^+/K^+-ATPase transporters normalized to intestinal mass (A) and nucleic acid content (B). There were significantly fewer Na^+/K^+-ATPase transporters in the intestine of aestivating animals relative to the intestine of active ones. Molecular activity of the Na^+/K^+-ATPase pumps was 2–3 times higher in aestivating tissues irrespective of whether data were normalized to tissue mass (C) or nucleic acid content (D). Results are presented as means ± s.e.m. Asterisks denote a significant difference from active animals at $P<0.05$.

Diamond, 1995; Secor, 2005; Secor, 2008a). Sit-and-wait foraging snakes and some infrequently feeding frogs may experience a reduction in nutrient uptake capacity of over 90% during interdigestive periods (Secor et al., 1994; Secor and Diamond, 1995; Secor, 2005). The 50% reduction in intestinal nutrient uptake capacity observed in aestivating *C. alboguttata* is, while perhaps somewhat more conservative, still consistent with these other studies.

Regulation of L-proline uptake rates by the SI of aestivating *C. alboguttata* might occur through changes in the activity of energetically expensive nutrient transporters or *via* less energetically expensive (passive) transport (Secor and Diamond, 1995). Traditional models suggest that most active nutrient transport systems are Na^+ dependent, while Na^+-independent uptake generally reflects that uptake which occurs passively *via* diffusion and may represent a less energetically expensive mechanism to absorb nutrients (Secor and Diamond, 1995). In *C. alboguttata* Na^+-dependent uptake comprised approximately 30% of total L-proline uptake by the SI. During aestivation, however, the amount of uptake occurring *via* Na^+-independent pathways increased markedly. An increase in the proportion of L-proline uptake occurring *via* Na^+-independent pathways might reflect a less energetically expensive means of maintaining the nutrient transport capacity of the tissue. Until recently, Na^+-independent nutrient transport in the vertebrate gut was considered to occur largely through diffusion. However, in pythons and hibernating ground squirrels, saturable uptake kinetics suggests that energy-consuming non-Na^+-dependent transport systems may be of significant importance in non-Na^+-dependent L-proline uptake by the SI (Carey and Sills, 1992; Carey, 1990; Secor and Diamond, 1995). Whether the increase in the amount of L-proline taken up *via* Na^+-independent pathways in *C. alboguttata* reflects diffusion or other carrier activities is unclear.

Energy costs of the SI during aestivation

During aestivation, *C. alboguttata* depress whole animal resting metabolic rate relative to standard metabolic rate by over 80% (Kayes et al., 2009). The transition from an active state to a hypometabolic or dormant state where metabolic rate (a measure of ATP turnover) may be significantly reduced requires a rebalancing of the rates of both ATP-producing and ATP-utilizing reactions (Hochachka, 1986a). Given the high energy costs associated with the maintenance of a functional SI (McBride and Kelly, 1990), we hypothesized that these costs should be down-regulated during aestivation when animals are not feeding. In aestivating *C. alboguttata*, however, the mass-specific oxygen consumption rate of the SI was not different from that of active animals although, as previously discussed, changes in absorptive cell size (Cramp et al., 2005) probably affect the relative number of cells per unit of tissue mass. Normalizing oxygen consumption rates to DNA content revealed that the intestinal cells of aestivating *C. alboguttata* were less metabolically active than those of active animals.

In the intestinal epithelium, protein synthesis and the activity of the Na^+/K^+-ATPase pump contribute to a substantial portion of total energy consumption by the tissue (Kelly and McBride, 1990; McBride and Kelly, 1990; Kelly et al., 1991; Cant et al., 1996). These two cellular processes are critical for the absorption of nutrients from the intestinal lumen during digestion and, consequently, changes in the intake of food markedly influence the rates of these processes and their related rates of oxygen consumption (Murray and Wild, 1980; McBride and Milligan, 1985a; McBride and Milligan, 1985b). In actively digesting *C. alboguttata*, protein synthesis and Na^+/K^+-ATPase activity, together, contributed to approximately 40% of cellular oxygen consumption. A substantial proportion of the remaining oxygen consumption rate is likely to be associated with the digestion, absorption and processing of food. In aestivating *C. alboguttata* oxygen consumption rates related to protein synthesis and Na^+/K^+-ATPase activity were not different from those of active animals; hence, the reduction in total cellular oxygen consumption rate observed in this study is likely to reflect the cessation of digestion, absorption and the processing of food in aestivating frogs.

The activities of the Na^+/K^+-ATPase transporter facilitate the maintenance of a gradient of Na^+ and K^+ across epithelial cell membranes, thereby providing the electrochemical driving force for Na^+-coupled nutrient uptake. During food deprivation, Na^+/K^+-ATPase activity often significantly declines in the intestine (Murray and Wild, 1980; McBride and Milligan, 1985b; Lucas-Teixeira et al., 2000). In addition, Na^+/K^+-ATPase activity has

been shown to decline in some other tissues of hypometabolic animals (Hochachka, 1986b; MacDonald and Storey, 1999; Ramnanan and Storey, 2006). In contrast, despite an overall reduction in the number of Na^+/K^+-ATPase transporters, overall Na^+/K^+-ATPase activity was maintained at active levels in the intestine of aestivating *C. alboguttata* through an increase in the molecular turnover rate of individual transporters. This finding is consistent with that of hibernating ground squirrels, in which intestinal Na^+/K^+-ATPase activity is maintained at active levels during hibernation (Carey and Martin, 1996). A reduction in the number of functional Na^+/K^+-ATPase pumps in the SI of aestivating *C. alboguttata* may be the result of a reduction in intestinal cell surface area (Cramp et al., 2005) and hence in order to maintain ionic gradients, the molecular activity of individual Na^+/K^+-ATPase transporters must be increased. A change in the molecular turnover rate of individual Na^+/K^+-ATPase transporters resulting in increased Na^+/K^+-ATPase activity has been described in several other instances including in the erythrocytes of cold-acclimated trout (Raynard and Cossins, 1991) and in the SI of cold-acclimated goldfish (Smith and Ellory, 1971). A change in the turnover rate of Na^+/K^+-ATPase transporters has been largely ascribed to changes in cell membrane phospholipid composition (Smith and Ellory, 1971; Raynard and Cossins, 1991; Else et al., 1996; Hulbert, 2007). Whether a change in membrane composition facilitates an increase in the molecular turnover rate of Na^+/K^+-ATPase transporters in the SI of aestivating *C. alboguttata* is as yet unknown.

During aestivation and hibernation, protein synthesis rates in many tissues decline during the hypometabolic period (Fuery et al., 1998; Pakay et al., 2002; Storey and Storey, 2007). Fasting has also been shown to affect rates of protein synthesis in the gut of some animals (McNurlan et al., 1979; Kelly et al., 1993; Samuels et al., 1996). In the intestine of many animals protein biosynthesis can account for up to 30% of the rate of oxygen consumption of the tissue (McBride and Kelly, 1990) and thus is a likely target for suppression especially in metabolically depressed organisms. In aestivating *C. alboguttata*, the rate of oxygen uptake associated with protein synthesis in the SI was equivalent to that of active frogs. Although we have no specific data on the rates of protein synthesis in the intestine of aestivating frogs, the maintenance of oxygen consumption rates associated with protein synthesis at the same level as active frogs is consistent with the maintenance of this process during aestivation.

CONCLUSIONS

The maintenance of energetically expensive cellular processes in the SI (including L-proline transport rates, Na^+/K^+-ATPase activity and protein synthesis) implies that they serve an important function during aestivation, despite the lack of dietary input. Even during fasting, the intestinal lumen is rarely devoid of nutrients as substrates may enter the gut lumen *via* passive diffusion along a concentration gradient from the gut tissue, or from cell sloughing (Ferraris and Carey, 2000). Hence, maintenance of a level of nutrient transport capacity during dormancy might ensure that these nutrients can be reclaimed by the gut, rather than being lost. In hibernating and aestivating animals whose survival rests on their ability to conserve endogenous fuels during dormancy, the loss of valuable nutrients would impact on the survival potential of the animal. Furthermore, the constant stimulation of nutrient transporter systems during hibernation/aestivation may be necessary to maximize absorptive efficiency during re-feeding so as to rapidly restock endogenous fuel stores (Carey, 1990).

Despite the loss of L-proline uptake capacity, the assimilation efficiency of re-feeding *C. alboguttata* is not affected by aestivation (Cramp and Franklin, 2003). In a recent review of python gastrointestinal regulation, it was noted that rapid changes in post-feeding nutrient uptake capacity may arise through a concomitant increase in the available surface area for absorption (microvilli lengthening) (Secor, 2008b). While *C. alboguttata* also experiences a reduction in intestinal absorptive surface area (i.e. villus and microvillus lengths) during aestivation (Cramp et al., 2005), re-feeding restores intestinal morphology within 2 days (Cramp and Franklin, 2005). Therefore it is possible that the rapid restoration of intestinal morphology in conjunction with the maintenance of a minimum level of nutrient transport capacity serves to ensure that absorption efficiency is maximized from the first meal post-aestivation. In future studies, we hope to investigate this hypothesis by examining the above cellular processes in re-feeding animals post-aestivation.

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