Journal of Fish Biology (2008) **73,** 608–622
doi:10.1111/j.1095-8649.2008.01955.x, available online at http://www.blackwell-synergy.com

Enzymatic and mitochondrial responses to 5 months of aerial exposure in the slender lungfish *Protopterus dolloi*

J. F. Staples*†, M. Kajimura‡§, C. M. Wood‡‖, M. Patel‡, Y. K. Ip¶ and G. B. McClelland‡

Department of Biology, University of Western Ontario, London ON, N6A 5B7 Canada, ‡Department of Biology, McMaster University, Hamilton ON, L8S 4K1 Canada, §Biological Laboratory, Faculty of Education, Wakayama University, Sakaedani 930, Wakayama 640-8510, Japan, ‖Division of Marine Biology and Fisheries, Rosenstiel School of Marine and Atmospheric Science and ¶Department of Biological Sciences, National University of Singapore, Science Dive 4, 117543, Singapore

(Received 27 September 2007, Accepted 29 April 2008)

Mitochondrial respiration and activities of key metabolic enzymes from liver and white skeletal muscle were compared between control aquatic slender lungfish *Protopterus dolloi*, and those exposed to air for 5 months. Activities of citrate synthase, glycogen phosphorylase, phosphofructokinase and pyruvate kinase in liver were not affected by air-exposure. In muscle, air-exposure reduced citrate synthase and pyruvate kinase activities (relative to tissue wet mass) by 63 and 50%, respectively. Liver carnitine palmitoyl transferase activity (relative to mitochondrial protein) decreased by half following air-exposure, but there was no change in muscle. In mitochondria isolated from muscle, state 3 and state 4 respiration were reduced by 74 and 89%, respectively following air-exposure, but liver mitochondria were not affected. In liver, air-exposure increased activities of ornithine-urea cycle enzymes including glutamine synthase, carbamoyl-phosphate synthase III and arginase, by 1·9- to 4·2-fold. Carbamoyl-phosphate synthase III activity could not be detected in muscle, indicating that urea is not synthesized in this tissue. These data suggest that skeletal muscle metabolism is downregulated in air-exposure, conserving energy and protein during a period when the animals cannot forage. In contrast, ATP production capacities in the liver are maintained, and this may permit expensive urea biosynthesis to continue during aerial exposure.

Key words: aestivation; Dipnoi; glycolysis; ornithine-urea cycle; oxidative phosphorylation.

INTRODUCTION

The habitat of the African slender lungfish *Protopterus dolloi* Boulenger is subject to seasonal drought and, as freshwater recedes, many physiological functions may become compromised. Aestivation comprises a suite of strategies that can help lungfishes cope with the challenges posed by environmental desiccation,

†Author to whom correspondence should be addressed. Tel.: +1 519 661 4057; fax: +1 519 661 3935; email: jfstaple@uwo.ca

allowing survival for several months without water or food. African lungfishes aestivate within a mucous cocoon secreted from the skin, which, after drying, provides some protection from water loss. In the marble African lungfish *Protopterus aethiopicus* Heckel and the West African lungfish *Protopterus annectens* (Owen) this cocoon is formed after the animal has burrowed into the mud at the bottom of a drying water source or inside a muslin sac within a laboratory (Fishman *et al.*, 1986). Although aestivating *P. dolloi* are not known to secrete a cocoon in the wild (Brien *et al.*, 1959; Greenwood, 1986), cocoon formation can be induced in the laboratory simply by withdrawing most of the water from aquaria (Chew *et al.*, 2004; Wood *et al.*, 2005), making it a very tractable experimental model. In the current study, the animals were the same as those used by Wilkie *et al.* (2007), where a cocoon was formed over the entire body except the ventral surface, and movement virtually ceased. As this condition may differ from aestivation it is referred to as 'air-exposure'.

Foraging is not possible when confined to a cocoon and, to conserve limited energy stores, it might be predicted that aestivating lungfishes would reduce metabolic rates during this time. In *P. dolloi*, however, there was no detectable change in oxygen consumption rates after 30 days of air-exposure (S. F. Perry, pers. comm.). It is possible that more time is necessary for metabolic suppression to develop in this species: according to Fishman *et al.* (1986) it requires up to 50 days for aestivating *P. aethiopicus* to express noticeable reductions in metabolic rate during aestivation. This time course of metabolic suppression is similar to that seen in overwintering frogs (Donohoe *et al.*, 1998).

While whole-organism metabolism may decrease during prolonged air-exposure, individual tissues may respond differently. For example, there is little movement within cocoons, so a downregulation of skeletal muscle metabolism might be predicted. As skeletal muscle accounts for *c.* 60% of total body mass (Wilkie *et al.*, 2007), any metabolic suppression in this tissue would contribute greatly to organismal energy savings. On the other hand, air exposure results in a three-fold increase in urea production, substantial accumulation of urea in tissues and 70% decreases in ammonia production (Chew *et al.*, 2003). Urea synthesis is energetically expensive (1 mole of urea requires 5 moles of ATP-equivalents; Ip *et al.*, 2004), so the switch from ammonia to urea production during air exposure may require the maintenance of, if not an increase in, ATP production capacities in ureagenic tissues.

Similar states of metabolic suppression in other animals are often associated with significant alterations in mitochondrial properties. In hibernating mammals, liver mitochondrial respiration is reduced by up to 70% compared with the summer active state (Muleme *et al.*, 2006). In frogs overwintering under hypoxic conditions, a 75% suppression of whole-animal metabolism is accompanied by a significant decrease in state 4 (non-phosphorylating) respiration in mitochondria isolated from skeletal muscle (St-Pierre *et al.*, 2000). In aestivating snails a profound (84%) whole-animal metabolic suppression (Pedler *et al.*, 1996) is accompanied by a 30% suppression of hepatopancreas cell metabolism (Guppy *et al.*, 2000) and significant reductions in mitochondrial substrate oxidation (Bishop *et al.*, 2002). Therefore, one goal of the current study was to determine if downregulated mitochondrial metabolism occurs in *P. dolloi* after 5 months of air-exposure, and whether mitochondrial responses differ among tissues.

Aestivation is often associated with decreases in maximal activities of key metabolic enzymes, and a preference for lipid oxidation to help conserve carbohydrates stores and muscle protein (Storey, 2002). Another goal of this study was to compare the activities of enzymes involved in glycolysis, fatty acid oxidation and the Krebs cycle between the control aquatic and air exposure states.

The switch from ammonia to urea excretion during air-exposure involves increases in the activities of key ornithine-urea cycle (OUC) enzymes in the liver (Chew *et al.*, 2004), but skeletal muscle may also be an important source of urea production. Skeletal muscle is known to express key OUC enzymes such as carbamoyl-phosphate synthase III (CPSaseIII) in some ammonotelic teleosts (Korte *et al.*, 1997), ureotelic teleosts (Lindley *et al.*, 1999) and bowfin *Amia calva* L., an air-breathing holostean fish (Felskie *et al.*, 1998). Indeed skeletal muscle is the major site of CPSaseIII activity and urea production in Lake Magadi, Kenya, tilapia *Oreochromis alcalicus* (Hilgendorf) living in alkaline water (Lindley *et al.*, 1999). In the dogfish *Squalus acanthias* L., CPSaseIII and other OUC enzymes in skeletal muscle are upregulated after feeding, facilitating increased rates of urea production (Kajimura *et al.*, 2006). After 40 days of air-exposure in *P. dolloi*, urea concentrations in skeletal muscle are significantly elevated and liver OUC enzyme activities are significantly increased (Chew *et al.*, 2004). As far as is known, however, these enzymes have not been examined in skeletal muscle of lungfishes. Therefore, a further goal of this study was to examine the effect of air-exposure on OUC enzymes in skeletal muscle.

MATERIALS AND METHODS

ANIMALS AND EXPERIMENTAL DESIGN

Protopterus dolloi (mean mass 156 g, range 56–274 g) were obtained and housed as described by Wilkie *et al.* (2007). All experimental and holding procedures followed Canadian Council on Animal Care guidelines and were approved by the McMaster University Animal Care Committee.

All animals were held at 27° C. One group of animals (fully aquatic) remained in aquaria filled with water for the duration of the experiment, and ate blood worms three times weekly. Food was withheld from this control group for 3–5 days before experiments. Another group of animals were exposed to air by draining all but 20 ml of water from their 10 l aquaria. These animals were misted with water every 6 days to maintain humidity within the aquaria. These air-exposure conditions induced the formation of cocoons over all but the ventral surface within 1 week. Air-exposed animals survive under these conditions for up to 8 months (unpubl. obs.). In this study, air-exposed animals were sampled 5 months after the initial withdrawal of water. Control, fully aquatic animals were held for the same length of time, and sampled at the same time as air-exposed animals. Livers from three control animals and four air-exposed animals were sampled. Epaxial skeletal white muscle from five control and three air-exposed animals was also sampled.

MITOCHONDRIAL ISOLATION AND RESPIRATION

Fish were anaesthetized with 0·5 g of MS222 in 2 l of water (neutralized with NaOH) and then killed by a sharp blow to the head followed by decapitation. Tissues were rapidly excised and placed in the appropriate ice-cold buffer. Liver and white muscle mitochondria were isolated after gentle homogenization in isotonic buffers followed

by differential centrifugation, as previously described (Leary *et al.*, 1996, 2003). Mitochondria were stored on ice for 1–2 h before being used for respiration experiments.

Mitochondrial respiration was determined in 2 ml closed glass chambers maintained at 27° C. Rates of oxygen consumption were measured using polarographic oxygen electrodes (Rank Brother, Bottisham, Cambridge, U.K.) and recorded using computer data acquisition. Both liver (0·5 mg protein) and muscle mitochondria (0·75 mg protein) were incubated in a medium containing (in mM) 140 KCl, 20 HEPES, 5 Na_2HPO_4 and 1% (w/v) bovine serum albumin. Pyruvate (10 mM) and malate (1 mM), dissolved in the incubation medium, were added as oxidative substrates, and after stable state two respiration rates were established, 0·2 mM ADP was added to stimulate state 3 respiration. State 4 respiration was recorded after all ADP had been phosphorylated, and rates of oxygen consumption stabilized at lower levels. Respiratory control ratios (RCR) were calculated as state 3: state 4.

MEASUREMENTS OF ENZYME ACTIVITIES, PROTEIN, WATER AND ION CONTENT

Portions of liver and white muscle were frozen in liquid N_2 immediately after being removed from animals. These tissues were stored at −80° C before being assayed for enzyme activities. Tissues were homogenized in 10 volumes of homogenization buffer (in mM) 20 K_2HPO_4, 10 HEPES, 0·5 EDTA, 1 dithiothreitol, with 50% glycerol, and adjusted with NaOH to pH 7·5 at 27° C, as described previously (Kajimura *et al.*, 2006) followed by sonication. Homogenates were centrifuged at 8000 *g* for 20 min at 4° C. The supernatant was retained and stored at −80° C for up to a week until analysed with no loss of activity.

Activities of CPSaseIII, ornithine-citrulline transcarbomylase (OCT), glutamine synthase (GS) and arginase (ARG) were assayed using the methods of Kajimura *et al.* (2006). Activities of glycogen phosphorylase (GP, total), phosphofructokinase (PFK) and pyruvate kinase (PK) were assayed, as previously described (Storey, 1987), with optimal substrate and activator concentrations determined empirically. Citrate synthase (CS) was assayed as previously described for fish tissues (McClelland *et al.*, 2005). Aliquots of freshly isolated mitochondria were used for measurements of total carnitine palmitoyl transferase (CPT) activity, as previously described (Rodnick & Sidell, 1994). Briefly, the assay buffer consisted of (in mM) 20 HEPES, 40 KCl, 1 EGTA, 220 sucrose, 0·1 DTNB and 1 carnitine. The reaction was followed at 412 nm after the addition of 0·04 mM palmitoyl-CoA. Soluble protein content of tissue homogenates (stored at −80° C) and fresh mitochondrial preparations were determined using a dye-binding assay (Bio-Rad, Hercules, CA, U.S.A.).

For determination of muscle and liver ion concentrations, tissue samples were digested in 1 N HNO_3 (5:1 v/w) at 65° C for 48 h. After vortexing, a 2 ml aliquot was removed and centrifuged at 13 000 *g* for 10 min. The supernatant was then appropriately diluted and analysed for Na^+ and Cl^- as in Wilkie *et al.* (2007). To determine water content, tissue samples were weighed and dried at 65° C for up to 96 h until mass no longer changed.

DATA ANALYSIS

Tissue protein and ion contents, mitochondrial respiration rates, RCR and maximal enzyme activities were compared between control and air-exposed animals by *t*-tests ($\alpha = 0{\cdot}05$) for both liver and white skeletal muscle. Data were $\log_{10}$ transformed if *F*-tests indicated that the variances of the raw data from the two groups were not equal.

RESULTS

The soluble protein concentration of liver ($P > 0{\cdot}05$) and white muscle ($P > 0{\cdot}05$) did not differ between the control and air-exposure conditions (Table I).

TABLE I. Tissue ion, water and soluble protein concentrations from *Protopterus dolloi* held under fully aquatic conditions (control) or after 5 months of air exposure

	Liver				Muscle			
	Na^+ (μmol g^{-1} wm)	Cl^- (μmol g^{-1} wm)	Water (% wm)	Protein (mg souble protein g^{-1} wm)	Na^+ (μmol g^{-1} wm)	Cl^- (μmol g^{-1} wm)	Water (% wm)	Protein (mg souble protein g^{-1} wm)
Control	10·4 (10·3; 10·5)	15·0 (14·5; 15·5)	75·6 (74·9; 76·2)	73·4 ± 6·7	28·7 ± 4·8	20·6 ± 3·0	80·1 ± 0·6	38·6 ± 4·2
n	2	2	2	3	4	4	5	5
Air-exposed	9·4 ± 0·4	15·5 ± 7·3	79·3* (79·5; 79·1)	85·5 (83·7; 87·4)	28·5 ± 5·2	22·5 ± 4·4	85·3 ± 0·7*	26·0 ± 1·6
n	4	3	2	2	4	3	5	3

Values are mean ± S.E., except where sample size is (*n*) <3, where values are mean (range).
*Values are significantly greater when comparing control and air exposure treatments (*t*-test, $P < 0{\cdot}05$). Muscle water content data were calculated from Wilkie *et al.* (2007).
wm, wet mass.

In both white muscle ($P < 0{\cdot}001$) and liver ($P < 0{\cdot}05$), water content increased significantly following air-exposure, but there was no significant change in Na^+ or Cl^- concentration (Table I).

Activities of energy metabolism enzymes expressed relative to wet tissue mass, the most common method of reporting such data, are given in Table II (a). Since tissue water content was affected by air-exposure, but soluble protein was not (Table I), however, enzyme activities relative to tissue protein are also reported [Table II (b)]. The activity of CS, GP, PFK and PK in liver did not differ significantly between 5 month air-exposed and control aquatic animals whether expressed relative to wet mass or soluble protein. In white muscle, there was a trend towards decreased activity of CS, GP and PK following air-exposure. Although these decreases were only statistically significant for CS (63%, $P < 0{\cdot}05$) and PK (50%, $P < 0{\cdot}05$) when calculated relative to tissue wet mass, this pattern is also evident when activities are expressed relative to tissue protein. White muscle PFK activity tended to increase following air exposure, but only when activity was calculated relative to protein did this increase (2·8-fold) reach statistical significance ($P < 0{\cdot}05$).

Total CPT activity (CPTI and CPTII) was more than two-fold greater ($P < 0{\cdot}05$) in the liver mitochondria from control animals compared with air-exposed animals (Fig. 1). In contrast, muscle CPT did not differ substantially after prolonged air-exposure. CPT activity was inhibited by 50 μM malonyl-CoA by 57–61% in muscle and 71–74% in liver.

Livers from animals subjected to prolonged air exposure appeared bilious and were rinsed thoroughly in homogenization buffer to remove any bile contamination. In mitochondria isolated from liver neither state 3 nor state 4 respiration rates differed significantly between the control aquatic and air-exposure treatments [Fig. 2(a)]. Respiratory control ratios for control ($5{\cdot}9 \pm 1{\cdot}4$, mean ± S.E.) and air-exposed liver mitochondria ($3{\cdot}5 \pm 1{\cdot}5$) did not differ significantly ($P > 0{\cdot}05$).

In contrast to liver mitochondria, air exposure resulted in 74 and 89% decreases in muscle mitochondria state 3 and state 4 respiration rates, respectively [Fig. 2(b)]. Although state 4 declined more than state 3 after air exposure, respiratory control ratios did not differ significantly between the control ($3{\cdot}0 \pm 0{\cdot}2$, mean ± S.E.) and air-exposure conditions ($5{\cdot}6 \pm 2{\cdot}1$). Glutamate and malate (10 and 1 mM) and palmitoyl carnitine (1 mM) were not oxidized effectively, producing respiration rates less than half of those achieved with pyruvate and malate.

In the liver, long-term air exposure resulted in 1·9- to 4·2-fold increases in the activities of liver OUC enzymes when expressed relative to tissue wet mass [Table III (a)]. All of these increases were statistically significant, except for OCT ($P > 0{\cdot}05$), where there was considerable interindividual variability in the air-exposed group. In white muscle CPSaseIII activity could not be detected, and air-exposure did not significantly alter the activities of GS, OTC and ARG [Table III (a)]. These patterns were largely maintained even when enzyme activities were calculated relative to tissue protein, except that variability prevented any significant difference in liver ARG ($P > 0{\cdot}05$), and muscle GS decreased significantly after air-exposure [Table III (b)].

TABLE II. Maximal activities of key energy metabolism enzymes in liver and white skeletal muscle of *Protopterus dolloi* in the fully aquatic (control) state and after 5 months of air exposure

	Liver				Muscle			
	CS (units g^{-1} wm)	GP (units g^{-1} wm)	PFK (units g^{-1} wm)	PK (units g^{-1} wm)	CS (units g^{-1} wm)	GP (units g^{-1} wm)	PFK (units g^{-1} wm)	PK (units g^{-1} wm)
(a)								
Control	0·77 ± 0·09	0·19 ± 0·01	6·10 ± 1·80	30·20 ± 18·81	0·51 ± 0·06*	0·65 ± 0·28	1·40 ± 0·42	89·60 ± 14·70*
Air-exposed	0·80 ± 0·04	0·17 ± 0·06	5·40 ± 0·40	10·61 ± 0·52	0·19 ± 0·06	0·31 ± 0·09	2·51 ± 0·43	44·40 ± 3·22
	Liver				Muscle			
	CS (m units mg^{-1} protein)	GP (m units mg^{-1} protein)	PFK (m units mg^{-1} protein)	PK (m units mg^{-1} protein)	CS (m units mg^{-1} protein)	GP (m units mg^{-1} protein)	PFK (m units mg^{-1} protein)	PK (m units mg^{-1} protein)
(b)								
Control	10·4 ± 0·2	15·0 ± 3·7	81·8 ± 20·8	234·8 ± 163·3	13·9 ± 2·7	108·2 ± 36·5	35·2 ± 6·6	1138·7 ± 132·4
Air-exposed	9·4 ± 0·4	15·5 ± 7·3	63·6 ± 4·8	62·6 ± 5·2	7·6 ± 2·8	52·9 ± 14·7	99·8 ± 20·1*	854·4 ± 46·1

Values are means ± S.E.

CS, citrate synthase; GP, glycogen phosphorylase; PFK, phosphofructokinase; PK, pyruvate kinase; wm, wet mass. Sample sizes: control liver, 3; air-exposed liver, 4; control muscle, 5; air-exposed muscle, 3.

*Values that are significantly greater when comparing control and air-exposure treatments (*t*-test, $P < 0·05$).

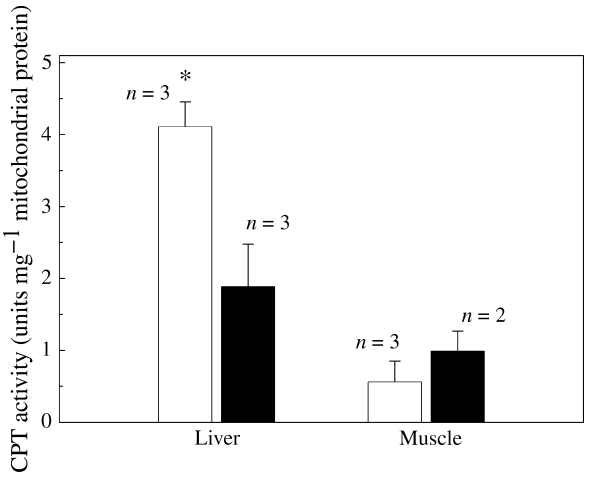

FIG. 1. Activity of total carnitine palmitoyl transferase (CPT1 + CPT2) in mitochondria isolated from liver and white skeletal muscle of *Protopterus dolloi* in the fully aquatic (control) state (□) and after 5 months of air exposure (■). Values are mean ± S.E. except for the muscle air-exposure group, where error bars represent range. *, Significantly greater (*t*-test, $P < 0{\cdot}05$). Sample sizes (*n*) are indicated.

DISCUSSION

After 5 months of air exposure in *P. dolloi*, body mass did not change (Wilkie *et al.*, 2007) and, although liver and muscle water content increased slightly, protein content did not change. Atlantic cod *Gadus morhua* L. starved for 16 weeks experience comparable tissue water gain, but lose significant tissue protein (Martinez *et al.*, 2003). Metabolic suppression during air exposure in lungfishes would conserve energy and muscle proteins. The present data suggest that glycolytic and oxidative metabolic capacities are reduced in skeletal muscle but not in liver, the main site of urea synthesis. The conclusions must remain tentative, however, due to small sample sizes resulting from limited animal availability.

Decreasing glycolysis during air exposure could conserve carbohydrates and reduce the need to catabolize muscle proteins for gluconeogenesis. Indeed in *P. aethiopicus* and *P. annectens*, liver and muscle glycogen are remarkably preserved after 6 months of aestivation (Janssens, 1964). Activity of GP did not change in either liver or muscle following air exposure (Table II) in agreement with liver data from aestivating *P. aethiopicus* and *P. annectens* (Janssens, 1964). White muscle PK activity from control animals is comparable to fully aquatic *P. aethiopicus* (Dunn *et al.*, 1983), but decreases significantly (when expressed relative to tissue wet mass) after air exposure. Muscle PFK activity actually increases after air exposure (when expressed relative to soluble protein). In other vertebrates PFK is inhibited by urea accumulation (Cowan & Storey, 2002) and decreases in pH (Hand & Somero, 1983), both of which occur during air exposure (Wilkie *et al.*, 2007) and aestivation (DeLaney *et al.*, 1977) in *P. dolloi* and *P. aethiopicus* so the apparent increase in muscle PFK may only offset these inhibitory effects. PFK is known to bind to cyctoskeletal elements

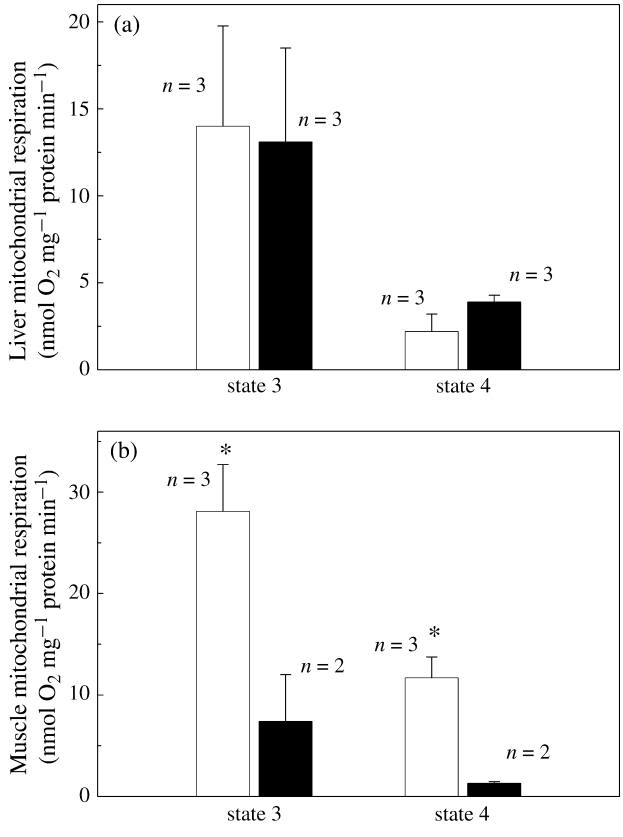

FIG. 2. Respiration of mitochondria isolated from (a) liver and (b) white skeletal muscle of *Protopterus dolloi* in the fully aquatic (control) state (□) and after 5 months of air exposure (■). Both state 3 and state 4 respiration rates are given. Values are mean ± S.E. except for the muscle air-exposure group, where error bars represent range. Sample sizes (*n*) are indicated beside the bars. *, Significantly greater (*t*-test, $P < 0{\cdot}05$).

and myofibrils (Su & Storey, 1995), perhaps facilitating glycolysis in hypometabolic states (Duncan & Storey, 1992). If PFK were to dissociate from the particulate fraction during air exposure, more PFK would remain in the supernatant after centrifugation, perhaps resulting in the increased total PFK activity observed in this study. Moreover, the glycolytic enzyme assays were performed under maximally activating conditions and because tissues were limited, it was not possible to determine the levels of active *v.* inactive forms of covalently regulated enzymes. For example, phosphorylation reduces liver GP activity by 78% in hibernating mammals (Storey, 1987). The present data, therefore, undoubtedly overestimate *in vivo* activities of GP, PFK and PK.

Muscle CPT was not altered by air-exposure, but in liver it decreased by 50%, suggesting a downregulation of maximal β-oxidation capacity. By contrast, fasting in most vertebrates enhances fat oxidation, sparing carbohydrate and protein stores. In both liver and muscle, CPT was inhibited by malonyl-CoA, so if fatty acid synthesis decreases during air exposure and levels of

TABLE III. Maximal activities of ornithine-urea cycle enzymes from liver and skeletal muscle of *Protopterus dolloi* held under fully aquatic conditions (control) or after 5 months of air exposure

	Liver				Muscle			
	GS (units g^{-1} wm)	OCT (units g^{-1} wm)	CPSaseIII (units g^{-1} wm)	ARG (units g^{-1} wm)	GS (units g^{-1} wm)	OCT (units g^{-1} wm)	CPSaseIII (units g^{-1} wm)	ARG (units g^{-1} wm)
(a)								
Control	0·08 ± 0·06	25·72 ± 6·13	0·06 ± 0·02	36·42 ± 5·42	0·09 ± 0·01	0·48 ± 0·12	ND	0·38 ± 0·11
Air-exposed	0·25 ± 0·04*	67·31 ± 16·74	0·27 ± 0·03*	69·83 ± 5·33*	0·02 ± 0·01	0·20 ± 0·02	ND	0·60 ± 0·34
	Liver				Muscle			
	GS (m units mg^{-1} protein)	OCT (m units mg^{-1} protein)	CPSaseIII (m units mg^{-1} protein)	ARG (m units mg^{-1} protein)	GS (m units mg^{-1} protein)	OCT (m units mg^{-1} protein)	CPSaseIII (m units mg^{-1} protein)	ARG (m units mg^{-1} protein)
(b)								
Control	0·98 ± 0·68	342·23 ± 54·51	0·85 ± 0·21	514·99 ± 121·2	2·44 ± 0·30*	13·53 ± 4·21	ND	10·71 ± 4·01
Air-exposed	2·81 ± 0·29*	675·74 ± 161·4	3·10 ± 0·27*	749·28 ± 79·6	0·94 ± 0·13	8·62 ± 0·9	ND	24·50 ± 15·12

Values are mean ± S.E.

ARG, arginase; CPSaseIII, carbamoyl-phosphate synthase III; GS, glutamine synthase; ND, below detection limit; OCT, ornithine-citrulline transcarbomylase; wm, wet mass (sample sizes see Table II).

*Values that are significantly greater (*t*-test).

malonyl-CoA levels decline, fatty acid oxidation rates could be maintained despite lower maximal CPT activities. It would be interesting to compare tissue concentrations of malonyl-CoA and activities of acetyl-CoA carboxylase (the enzyme that synthesizes malonyl-CoA) between fully aquatic and air-exposed *P. dolloi*. Indeed in hibernating mammals, reduced acetyl-CoA carboxylase activity facilitates upregulated fat oxidation (Belke *et al.*, 1998).

Muscle CS in control fish was slightly lower than fully aquatic *P. aethiopicus* (Dunn *et al.*, 1983), but declined by 63% after air-exposure to a level much lower than aestivating *Lepidosiren paradoxa* Fitzinger (Mesquita-Saad *et al.*, 2002). During air-exposure there was very little apparent muscular activity. In rats, hind limb immobilization results in dramatic decreases in CS activity within 10 days (Booth, 1977). In contrast, mammalian hibernators appear to defend muscle CS activities (Wickler *et al.*, 1991) and power output (Harlow *et al.*, 2001; Lohuis *et al.*, 2007) despite extended periods of apparent inactivity. Decreased muscle CS may result from reduced mitochondrial volume density, or reduced CS activity of individual mitochondria, as seen in overwintering frogs (St-Pierre & Boutilier, 2001). This could be assessed by measuring CS in isolated mitochondria, as opposed to whole tissue as reported here. Unfortunately, the low yield of mitochondria did not permit such measurements in the current study. Although liver CS was not affected by air-exposure (Table II), liver cytochrome *c* oxidase activity declines by three-fold in aestivating *P. dolloi* (Frick, 2007). The apparent conflict between these data sets may represent differences in regulation of Krebs cycle *v.* electron transport chain in hypometabolic states (perhaps by changes in inner mitochondrial membrane cardiolipin; Frick, 2007) or fundamental differences between air-exposure and aestivation.

Like the CS data, mitochondrial data suggest maintained liver oxidative capacity, but downregulated muscle metabolism. White muscle mitochondria from control animals had state 3 respiration rates that were *c.* 50% lower than a more active species, rainbow trout *Oncorhynchus mykiss* (Walbaum) (Leary *et al.*, 2003), but state 4 rates were comparable. After air-exposure state 3 and 4 rates were reduced by 74 and 89%, respectively [Fig. 2(b)]. Moreover, glutamate supported only very low mitochondrial respiration rates, suggesting that amino acids are not a preferred oxidative substrate, which would further preserve tissue protein. Due to limited availability of animals and tissue, the sample sizes for this experiment were rather small (control muscle $n = 3$, air-exposed muscle $n = 3$), so the conclusions must remain tentative. Nonetheless evidence from across many taxa suggests that mitochondrial metabolic suppression is a common strategy for energy conservation during extended periods of environmental stress, being employed in skeletal muscle of overwintering frogs (St-Pierre *et al.*, 2000), hepatopancreas of aestivating snails (Bishop *et al.*, 2002) and liver in hibernating ground squirrels (Barger *et al.*, 2003; Muleme *et al.*, 2006) and torpid Siberian hamsters (Brown *et al.*, 2007).

Despite increases in muscle and liver water content, neither Na^+ nor Cl^- concentrations in these tissues changed following air-exposure (Table I), suggesting that intracellular osmolarity did not change to any great extent. Although muscle urea content increases to 80 μmol g^{-1} wet mass following air exposure (Wilkie *et al.*, 2007), these concentrations are unlikely to affect

mitochondrial respiration (Anderson, 1986). Indeed RCR values (used to assess the quality of mitochondrial preparations) for air-exposed preparations did not differ from control preparations. These observations suggest that differences in muscle mitochondrial function reported in the current study are not related to osmotic effects or any other preparation artefact. In *P. aethiopicus*, however, plasma ions increase substantially (*e.g.* Na^+ increases *c.* 80%) and urea rise to >200 mM during aestivation (DeLaney *et al.*, 1977). These changes, if reflected in the intracellular fluid, would substantially affect enzymes and mitochondria. The prolonged air-exposure examined in this study may, therefore, represent a transition state during which metabolism is remodelled to prepare for the extended fasting and desiccation of aestivation.

During air exposure, the mitochondria of the largely inactive skeletal muscle probably operate *in vivo* closer to state 4 (respiration limited by ADP availability) than state 3 (excess ADP, substrate and oxygen, resulting in near-maximal respiration). Reduced state 4 respiration may result from decreased substrate oxidation, used to generate the proton motive force across the inner mitochondrial membrane, and proton leak, which dissipates the proton motive force. It would be interesting to compare the kinetics of proton leak (St-Pierre *et al.*, 2000; Brown *et al.*, 2007) between the control and air-exposure conditions.

Unlike muscle, the present data suggest that liver retains metabolic capacities even after 5 months of air exposure. The increase in liver OUC enzyme activities (Table III) confirm previous work on *P. dolloi* (Chew *et al.*, 2004), *P. annectens* and *P. aethiopicus* (Loong *et al.*, 2005). Upregulation of these liver enzymes may facilitate the 3·8-fold increased rate of urea synthesis observed by Chew *et al.* (2004) during 40 days of similar air exposure. Given the high metabolic cost of urea synthesis (Ip *et al.*, 2004), it was predicted that aerobic capacities would not be downregulated in liver to the same degree as in skeletal muscle. In addition to the retained liver CS activity (Table II), pyruvate respiration rates of intact liver mitochondria were maintained [Fig. 2(a)], perhaps ensuring sufficient ATP production capacity for urea biosynthesis.

This study is the first, as far as is known, to assay for OUC enzyme activity in lungfish skeletal muscle. There was no detectable CPSaseIII activity in skeletal muscle suggesting that, unlike *O. alcalicus* (Lindley *et al.*, 1999) or *S. acanthias* (Kajimura *et al.*, 2006), skeletal muscle is not an important source of urea production in *P. dolloi*, even during prolonged air exposure. After 5 months of air exposure urea levels in skeletal muscle rise to 80 μmol of urea g^{-1} (Wilkie *et al.*, 2007). This ability of skeletal muscle to accumulate urea, as opposed to ammonia, may be beneficial because of its lower toxicity. At high concentrations, however, urea inhibits enzymes such as PFK and glutamate dehydrogenase, especially in the presence of high KCl (Grundy & Storey, 1994) without counteracting solutes such as methylamines (Storey, 2002). Following 5 months of air-exposure in *P. dolloi*, neither trimethylamine oxide nor trimethylamine in plasma could be detected (Wilkie *et al.*, 2007).

In summary, this study shows that 5 months of air exposure within a cocoon resulted in a significant downregulation of metabolic capacities in skeletal muscle, but not liver. Reducing skeletal muscle metabolism would conserve energy and protein during a period when the animal cannot forage. The maintenance of liver ATP-synthesizing capacity may help to power increased rates of urea

synthesis in the air-exposed *P. dolloi*, an effect which was mirrored by substantial increases in activities of several OUC enzymes in liver. By contrast, the data indicate that skeletal muscle does not synthesize urea, even after air-exposure. These tissue-specific responses allow *P. dolloi* to reduce metabolism and avoid the accumulation of toxic waste products, permitting survival in this unique environment.

This study was supported by Discovery Grants from the Natural Sciences and Engineering Research Council (Canada) to J.F.S., G.B.M. and C.M.W., and the Canada Foundation for Innovation Awards to G.B.M. and C.M.W. The Canada Research Chair Program also provides support to C.M.W. G.B.M. is supported by an Early Researcher Award from the Ontario Ministry for Innovation. M.K. was supported by a Grant from JSPS Research Fellowships for Young Scientists. We would like to thank M. P. Wilkie, F. Galvez, R. W. Smith and S. Nadella for their assistance with handling animals and obtaining tissues.

References

Anderson, P. M. (1986). Effects of urea, trimethylamine oxide, and osmolality on respiration and citrulline synthesis by isolated hepatic mitochondria from *Squalus acanthias*. *Comparative Biochemistry and Physiology B* **85,** 783–788.

Barger, J., Brand, M. D., Barnes, B. M. & Boyer, B. B. (2003). Tissue-specific depression of mitochondrial proton leak and substrate oxidation in hibernating arctic ground squirrels. *American Journal of Physiology* **284,** R1306–R1313.

Belke, D. D., Wang, L. C. H. & Lopaschuk, G. D. (1998). Acetyl-CoA carboxylase control of fatty acid oxidation in hearts from hibernating Richardson's ground squirrels. *Biochimica et Biophysica Acta* **1391,** 25–36.

Bishop, T., St-Pierre, J. & Brand, M. D. (2002). Primary causes of decreased mitochondrial oxygen consumption during metabolic depression in snail cells. *American Journal of Physiology* **282,** R372–R382.

Booth, F. W. (1977). Time course of muscular atrophy during immobilization of hindlimbs in rats. *Journal of Applied Physiology* **43,** 656–661.

Brien, P., Poll, M. & Boiullon, J. (1959). Ethology de la reproduction de *Protopterus dolloi*. *Annals of the Museum of the Royal Belgian Congo* **71,** 3–21.

Brown, J. C. L., Gerson, A. R. & Staples, J. F. (2007). Mitochondrial metabolism during daily torpor in the dwarf Siberian hamster: the role of active regulated changes and passive thermal effects. *American Journal of Physiology* **293,** R1833–R1845.

Chew, S. F., Ong, T. F., Ho, L., Tam, W. L., Loong, A. M., Hiong, K. C., Wong, W. P. & Ip, Y. K. (2003). Urea synthesis in the African lungfish *Protopterus dolloi* – hepatic carbamoyl phosphate synthetase III and glutamine synthetase are upregulated by 6 days of aerial exposure. *Journal of Experimental Biology* **206,** 3615–3624.

Chew, S. F., Chan, N. K., Loong, A. M., Hiong, K. C., Tam, W. L. & Ip, Y. K. (2004). Nitrogen metabolism in the African lungfish (*Protopterus dolloi*) aestivating in a mucus cocoon on land. *Journal of Experimental Biology* **207,** 777–786.

Cowan, K. J. & Storey, K. B. (2002). Urea and KCl have differential effects on enzyme activities in liver and muscle of estivating versus nonestivating species. *Biochemistry and Cell Biology* **80,** 745–755.

DeLaney, R. G., Lahiri, S., Hamilton, R. & Fishman, P. (1977). Acid-base balance and plasma composition in the aestivating lungfish (*Protopterus*). *American Journal of Physiology* **232,** R10–R17.

Donohoe, P. H., West, T. G. & Boutilier, R. G. (1998). Respiratory, metabolic, and acid-base correlates of aerobic metabolic rate reduction in overwintering frogs. *American Journal of Physiology* **274,** R704–R710.

Duncan, J. A. & Storey, K. B. (1992). Subcelluar enzyme binding and the regulation of glycolysis in anoxic turtle brain. *American Journal of Physiology* **262,** R517–R523.

Dunn, J. F., Hochachka, P. W., Davison, W. & Guppy, M. (1983). Metabolic adjustments to diving and recovery in the African lungfish. *American Journal of Physiology* **245,** R651–R657.
Felskie, A. K., Anderson, P. M. & Wright, P. A. (1998). Expression and activity of carbamoyl phosphate synthetase III and ornithine urea cycle enzymes in various tissues of four fish species. *Comparative Biochemistry and Physiology B* **119,** 355–364.
Fishman, A. P., Pack, A. I., Delaney, R. C. & Galante, R. J. (1986). Estivation in *Protopterus*. *Journal of Morphology (Supplement)* **1,** 237–248.
Frick, N. (2007). Phospholipid and fatty acid modulations of mitochondrial membranes and correlations with cytochrome c oxidase activity in aestivating lungfish (Abstract). *Comparative Biochemistry and Physiology A* **148,** S155,
Greenwood, P. H. (1986). The natural history of African lungfishes. *Journal of Morphology* (*Supplement*) **1,** 163–179.
Grundy, J. E. & Storey, K. B. (1994). Urea and salt effects on enzymes from estivating and non-estivating amphibians. *Molecular and Cellular Biochemistry* **131,** 9–17.
Guppy, M., Reeves, D. C., Bishop, T., Withers, P., Buckingham, J. A. & Brand, M. D. (2000). Intrinsic metabolic depression in cells isolated from the hepatopancreas of estivating snails. *FASEB Journal* **14,** 999–1004.
Hand, S. C. & Somero, G. N. (1983). Phosphofructokinase of the hibernator *Citellus beecheyi*: temperature and pH regulation of activity via influences on the tetramer-dimer equilibrium. *Physiological Zoology* **56,** 380–388.
Harlow, H. J., Lohuis, T., Beck, T. D. I. & Iaizzo, P. A. (2001). Muscle strength in overwintering bears. *Nature* **409,** 997.
Ip, Y. K., Chew, S. F. & Randall, D. J. (2004). Five tropical air-breathing fishes, six different strategies to defend against ammonia toxicity on land. *Physiological and Biochemical Zoology* **77,** 768–782.
Janssens, P. A. (1964). The metabolism of the aestivating African lungfish. *Comparative Biochemistry and Physiology* **11,** 105–117.
Kajimura, M., Walsh, P. J., Mommsen, T. P. & Wood, C. M. (2006). The dogfish shark (*Squalus acanthias*) increases both hepatic and extrahepatic ornithine urea cycle enzyme activities for nitrogen conservation after feeding. *Physiological and Biochemical Zoology* **79,** 602–613.
Korte, J. J., Salo, W. L., Cabrera, V. M., Wright, P. A., Felskie, A. K. & Anderson, P. M. (1997). Expression of carbamoyl-phosphate synthetase III mRNA during the early stages of development and in muscle of adult rainbow trout (*Oncorhynchus mykiss*). *Journal of Biological Chemistry* **272,** 6270–6277.
Leary, S. C., Barton, K. N. & Ballantyne, J. S. (1996). Direct effects of 3,5,3′-triiodothyronine and 3,5-diiodothyronine on mitochondrial metabolism in the goldfish *Carassius auratus*. *General and Comparative Endocrinology* **104,** 61–66.
Leary, S. C., Lyons, C. N., Rosenberger, A. G., Ballantyne, J. S., Stillman, J. & Moyes, C. D. (2003). Fiber-type differences in muscle mitochondrial profiles. *American Journal of Physiology* **285,** R817–R826.
Lindley, T. E., Scheiderer, C. L., Walsh, P. J., Wood, C. M., Bergman, H. L., Bergman, A. L., Laurent, P., Wilson, P. & Anderson, P. M. (1999). Muscle as the primary site of urea cycle enzyme activity in an alkaline lake-adapted tilapia, *Oreochromis alcalicus grahami*. *Journal of Biological Chemistry* **274,** 29858–29861.
Lohuis, T. D., Harlow, H. J., Beck, T. D. & Iaizzo, P. A. (2007). Hibernating bears conserve muscle strength and maintain fatigue resistance. *Physiological and Biochemical Zoology* **80,** 257–269.
Loong, A. M., Hiong, K. C., Lee, S. M. L., Wong, W. P., Chew, S. F. & Ip, Y. K. (2005). Ornithine-urea cycle and urea synthesis in African lungfishes, *Protopterus aethiopicus* and *Protopterus annectens*, exposed to terrestrial conditions for six days. *Journal of Experimental Zoology* **303A,** 354–365.
Martinez, M., Guderley, H., Dutil, J.-D., Winger, P. D., He, P. & Walsh, S. J. (2003). Condition, prolonged swimming performance and muscle metabolic capacities of cod *Gadus morhua*. *Journal of Experimental Biology* **206,** 503–511.

McClelland, G. B., Dalziel, A. C., Fragoso, N. M. & Moyes, C. D. (2005). Muscle remodeling in relation to blood supply: implications for seasonal changes in mitochondrial enzymes. *Journal of Experimental Biology* **208,** 515–522.

Mesquita-Saad, L. S. B., Leitão, M. A. B., Paula-Silvan, M. N., Chippari-Gomes, A. R. & Almeida-Val, V. M. F. (2002). Specialized metabolism and biochemical suppression during aestivation in the extant South American lungfish – *Lepidosiren paradoxa*. *Brazilian Journal of Biology* **62,** 495–501.

Muleme, H. M., Walpole, A. C. & Staples, J. F. (2006). Mitochondrial metabolism in hibernation: metabolic suppression, temperature effects, and substrate preferences. *Physiological and Biochemical Zoology* **79,** 474–483.

Pedler, S., Fuery, C. J., Withers, P. C., Flanigan, J. & Guppy, M. (1996). Effectors of metabolic depression in an estivating pulmonate snail (*Helix aspersa*): whole animal and in vitro tissue studies. *Journal of Comparative Physiology B* **166,** 375–381.

Rodnick, K. J. & Sidell, B. D. (1994). Cold acclimation increases carnitine palmitoyltransferase I activity in oxidative muscle of striped bass. *American Journal of Physiology* **266,** R405–R412.

Storey, K. B. (1987). Regulation of liver metabolism by enzyme phosphorylation during mammalian hibernation. *Journal of Biological Chemistry* **262,** 1670–1673.

Storey, K. B. (2002). Life in the slow lane: molecular mechanisms of estivation. *Comparative Biochemistry and Physiology A* **133,** 733–754.

St-Pierre, J. & Boutilier, R. G. (2001). Aerobic capacity of frog skeletal muscle during hibernation. *Physiological and Biochemical Zoology* **74,** 390–397.

St-Pierre, J., Brand, M. D. & Boutilier, R. G. (2000). The effect of metabolic depression on proton leak rate in mitochondria from hibernating frogs. *Journal of Experimental Biology* **203,** 1469–1476.

Su, J. Y. & Storey, K. B. (1995). Phosphofructokinase binding to myofibrils in fish muscle: influences of ionic strength and metabolite levels on enzyme complex formation. *Biochemistry and Molecular Biology International* **35,** 781–791.

Wickler, S. J., Hoyt, D. F. & Breukelen, F. V. (1991). Disuse atrophy in hibernating golden-mantled ground squirrel, *Spermophilus lateralis*. *American Journal of Physiology* **261,** R1214–R1217.

Wilkie, M. P., Morgan, T. P., Galvez, F., Smith, R. W., Kajimura, M., Ip, Y. K. & Wood, C. M. (2007). The African lungfish (*Protopterus dolloi*): ionoregulation and osmoregulation in a fish out of water. *Physiological and Biochemical Zoology* **80,** 99–112.

Wood, C. M., Walsh, P. J., Chew, S. F. & Ip, Y. K. (2005). Greatly elevated urea excretion after air-exposure appears to be carrier mediated in the slender lungfish (*Protopterus dolloi*). *Physiological and Biochemical Zoology* **78,** 893–907.