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Surviving the drought: burrowing frogs save energy by increasing mitochondrial coupling

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SUMMARY

During dormancy energy conservation is a key priority and as such dormant animals undergo a major metabolic depression to conserve their limited endogenous fuel supplies. Mitochondrial coupling efficiency, the efficiency with which mitochondria convert oxygen into ATP, significantly affects aerobic metabolism and thus to maximise energy savings during dormancy it has been hypothesised that coupling efficiency should increase. However, previous studies have shown coupling efficiency to be maintained or even to decrease. In this study we measured state 3 and state 4 mitochondrial respiration in the muscle of the burrowing frog, *Cyclorana alboguttata* and calculated the respiratory control ratio as a measure of coupling efficiency. After 7 months in aestivation, *C. alboguttata* significantly reduced oxygen consumption of isolated mitochondria by 83% and, unlike other dormant animals, the frogs appeared to decrease rates of proton leak to a greater extent than ATP synthesis, consistent with an increase in mitochondrial coupling efficiency. The significant energy savings observed at the mitochondrial level were reflected at higher levels of biological organisation, with tissue oxygen consumption depressed by as much as 81% and whole animal metabolic rate by 82%. *Cyclorana alboguttata* can survive in a dormant state for several years and we propose the hypothesis that energy efficiency is increased during aestivation.

Key words: *Cyclorana alboguttata*, aestivation, down-regulation, mitochondria, oxygen consumption, skeletal muscle.

INTRODUCTION

In the absence of food, the key priority for an organism is the conservation of endogenous fuel supplies. Energy conservation in the absence of food is exemplified in animals that undergo periods of dormancy. Burrowing frogs are exceptional amongst vertebrates that undergo dormancy in that they can survive for several years in the absence of food and water, considerably longer than the most proficient mammalian hibernators. To achieve this, the frogs must undergo a major metabolic depression to conserve their limited endogenous fuel supplies. Because mitochondrial function underlies not only cellular but also tissue and whole organism aerobic metabolic rate, a reduction in mitochondrial oxygen consumption would result in significant energy savings during metabolic depression. Indeed, in hibernating and aestivating animals, rates of mitochondrial oxygen consumption decrease during dormancy (Pehowich and Wang, 1984; Gehnrich and Aprille, 1988; Brustovetsky et al., 1990; Brustovetsky et al., 1993; Martin et al., 1999; Bishop and Brand, 2000; Bishop et al., 2002).

Mitochondrial energy expenditure (oxygen consumption) is primarily associated with two major processes: ATP synthesis and proton leak across the inner mitochondrial membrane. Coupling efficiency is the proportion of the oxygen consumed to drive ATP synthesis compared with that driving proton leak, and can change with physiological state (Harper et al., 2008). Increasing mitochondrial coupling efficiency in conjunction with a decrease in overall oxygen consumption could theoretically further maximise energy savings at the mitochondrial level. However, this has yet to be shown in an animal that undergoes dormancy. Hibernating frogs (*Rana temporaria*) and aestivating snails (*Helix aspersa*) maintain levels of mitochondrial coupling during dormancy by decreasing proton leak in line with ATP synthesis, such that the relative proportion of oxygen diverted towards proton leak remains fixed (Bishop and Brand, 2000; Bishop et al., 2002; Boutilier and St-Pierre, 2002). Hibernating mammals, on the other hand, actually decrease mitochondrial coupling efficiency during dormancy, decreasing ATP synthesis but maintaining pre-hibernation levels of proton leak (Liu et al., 1969; Brustovetsky et al., 1989; Martin et al., 1999).

We were particularly interested in how mitochondrial respiration and coupling efficiency changed in an animal that undergoes more prolonged periods of metabolic depression at higher ambient temperatures, and the effect this may have on whole animal energy conservation The green striped burrowing frog, *Cyclorana alboguttata*, spends an average of 9–10 months a year in aestivation but is known to survive upwards of 3–5 years in dormancy during prolonged droughts. Here we report evidence of a potential increase in mitochondrial coupling efficiency during dormancy.

MATERIALS AND METHODS

Experimental animals

Adult *C. alboguttata* Günther 1867 (green-striped burrowing frog) were collected from flooded pools after heavy rains from the districts of Dalby and Theodore, Queensland, under Scientific Purposes Permit number WISP03572406 and brought to The University of Queensland. Frogs were housed in individual 5 l plastic containers lined with wet paper towels and fed once a week on crickets. Except for whole animal metabolic rate experiments, control animals were fed no less than 4 days prior to experimentation. Animals required to aestivate for long periods (7 months) were placed into 5 l containers filled with soil saturated with water. The soil was left to

naturally dry out, encouraging the frogs to burrow. Once burrowed the animals were left undisturbed for the duration of the aestivating period. All animals were maintained at 24±2°C with a 12 h:12 h light:dark regime for control animals and 24 h darkness for aestivating animals. All experiments were conducted under ethical clearance (AEC Approval numbers SIB/442/07/UQ and SIB/602/08/ARC).

Mitochondrial respiration

Mitochondrial respiration was measured in control (N=7) and 7 month aestivating (N=7) animals. Animals were killed *via* cranial and spinal pithing. Immediately after pithing, the muscles on the left hind limb were exposed by removal of the skin and dissected away from the bone. All hind limb muscles from a single frog were pooled to achieve suitable levels of mitochondria extraction. After weighing, the muscles were homogenized in 9 volumes of isolation medium (pH 7.3; 140 mmol l^{-1} KCl, 10 mmol l^{-1} EDTA, 5 mmol l^{-1} $MgCl_2$, 20 mmol l^{-1} Hepes, 0.5% BSA) and the homogenate was centrifuged at 1400 ***g*** for 5 min. The supernatant was collected and centrifuged at 9000 ***g*** for 7 min. After the second centrifugation, the supernatant was discarded and the mitochondrial pellet was resuspended in assay medium (pH 7.3; 140 mmol l^{-1} KCl, 20 mmol l^{-1} Hepes, 5 mmol l^{-1} Na_2HPO_4, 0.5% BSA) at a concentration of 100 µl per 0.1 g of tissue.

Respiration rate of the mitochondria was measured in a micro-respiration chamber (Mitocell MT200A, Strathkelvin Instruments, North Lanarkshire, Scotland, UK) containing an oxygen electrode (1302 electrode, Strathkelvin Instruments) connected to an oxygen meter (OM 200, Cameron Instrument Company, Port Aransas, TX, USA). The oxygen meter was connected to a PowerLab (ADInstruments, Sydney, NSW, Australia) analog to digital converter and a computer. The software program CHART© (ADInstruments) was used to record and analyse the acquired data. All experiments were conducted at 24°C.

The resuspended mitochondria were diluted 1 in 2 (aestivators) or 1 in 5 (controls) in assay medium and 250 µl of mitochondrial solution was added to the respiration chamber. Malate (final concentration 5 mmol l^{-1}) and pyruvate (final concentration 2.5 mmol l^{-1}) were added to the chamber to initiate state 2 respiration. ADP was then added to the chamber and state 3 respiration (ATP synthesis) was recorded until the ADP was exhausted and the respiration state changed to state 4 (an estimate of proton leak). The ATP synthase inhibitor oligomycin (1 µg ml^{-1}) was then added to the chamber and respiration rate was recorded in the absence of oxidative phosphorylation. The respiratory control ratio (RCR – a measure of the degree of mitochondrial coupling) was calculated by dividing state 3 by state 4 respiration.

To determine protein concentration 50 µl of undiluted mitochondrial solution was resuspended in 1 ml of BSA-free buffer and centrifuged at 12,000 ***g*** for 10 min. The pellet was retained and this process repeated three times. The final pellet was resuspended in 50 µl of distilled water; 5 µl of this suspension was added to 250 µl of Bradford reagent (Sigma-Aldrich, Castle Hill, NSW, Australia) and absorbance measured at 595 nm. Protein concentrations of mitochondrial preparations were determined using bovine serum albumin standards.

The rate of oxygen consumption ($\dot{V}_{O_2}$) was calculated in µl O_2 min^{-1} mg^{-1} protein using the following equation:

$$\dot{V}_{O_2} = (M \times \beta \times V) / m , \quad (1)$$

where M is the absolute slope (mmHg min^{-1}), β is the solubility coefficient of the assay medium (µl O_2 l^{-1} $mmHg^{-1}$) (Johnston et al., 1994), V is volume of the chamber (l) and m is the mass of mitochondrial protein (mg).

Tissue metabolic rate

Tissue metabolic rate was measured in control (N=11) and 7 month aestivators (N=8). Animals were killed by cranial and spinal pithing. The liver was removed from the body cavity through an incision in the ventral wall. The gastrocnemius muscle was removed from the right leg. Immediately after extraction, the tissues were blotted on a paper towel and weighed before being placed in oxygenated McKenzies' frog Ringer solution (pH 7.4; 111 mmol l^{-1} NaCl, 2.5 mmol l^{-1} KCl, 1.8 mmol l^{-1} $CaCl_2 \cdot 2H_2O$, 1 mmol l^{-1} $MgCl_2$, 5 mmol l^{-1} Hepes, 10 mmol l^{-1} glucose) until slicing. After slicing, tissues were allowed to recover for 30 min before metabolic rate was measured.

Tissue metabolic rate was measured at 24°C by sealing tissue slices in 3 ml plastic syringes containing 2 ml of fully oxygenated McKenzies' frog Ringer solution. After the syringes were sealed, the oxygen concentration of fully oxygenated Ringer solution was measured using an oxygen meter (782 Oxygen Meter, Strathkelvin Instruments) connected to an oxygen electrode (1302 electrode, Strathkelvin Instruments) in an electrode chamber. Measurements were taken by passing 1 ml of Ringer solution through the chamber and over the electrode membrane. Sealed syringes were placed in a water bath at 24°C for 2 h after which time the oxygen concentration of the Ringer solution in the syringes was measured, as described above. After the final concentrations of oxygen in the syringes were measured, the tissue slices were removed from the syringes, blotted and weighed. The rate of oxygen consumption was calculated per gram of wet tissue mass (µl h^{-1} g^{-1}) and determined using the following equation:

$$\dot{V}_{O_2} = \frac{(V \times (\text{initial}O_2 - \text{final}O_2)) / t}{m} , \quad (2)$$

where V is volume of Ringer solution (l), m is the mass of the tissue slice (g) and t is the period during which the chamber was sealed (h).

Changes in the oxygen concentration of syringes containing partially deoxygenated Ringer solution and no tissue slices were also measured to account for diffusion of oxygen across the syringe. Oxygen diffusion across the syringe was found to be negligible.

During the tissue metabolic rate experiments, it was noticed that there was a large size difference in the livers of control and aestivating animals. To examine the effect this might have on an animal's metabolic rate, per gram oxygen consumption of liver slices was multiplied by the mass of the whole organ and then standardised to a 25 g animal, using the formula of Fuery et al. (Fuery et al., 1998):

$$\dot{V}_{O2(\text{mass adjusted})} = (25\,\text{g} / \text{animal mass})^{-0.25} \times \dot{V}_{O2} . \quad (3)$$

Whole animal metabolic rate

Mass specific whole animal metabolic rate was measured using closed box respirometry. Frogs were randomly assigned to one of two treatment groups, control (N=9) and aestivators (N=8). Aestivating animals were placed into 1000 ml plastic respirometry chambers lined with a wet paper towel, which was allowed to slowly dry out to induce the onset of aestivation. Respirometry chambers were kept in a dark, constant temperature (24°C) room for the duration of the experiment. During non-sampling periods chambers were left ajar to allow air flow. During sampling periods respirometers were sealed and a red light was used to minimize disturbance to the frogs.

At the end of 10 weeks, control and aestivator metabolic rates were measured. Control animals were weighed and placed in their chambers 24 h prior to the respirometer being sealed and were removed immediately following the final oxygen measurements. At the beginning of a sampling period respirometry chambers were sealed and initial air samples of the chamber were taken using a syringe *via* a 3-way tap. Final samples were taken any time from 8 to 24 h later, depending on the treatment group (longer for aestivators) and the state of aestivation.

The fractional content of O_2 and CO_2 of the samples was analysed by injecting the sample through a Drierite column to remove water vapour and into a gas analyzer (ADInstruments) connected to a PowerLab (ADInstruments) analog to digital converter and a computer. The software program CHART© was used to record and analyse the acquired data. Metabolic rates were calculated using the formulae of Vleck (Vleck, 1987):

$$\mathrm{RER} = \frac{F_{\mathrm{endCO_2}} - F_{\mathrm{startCO_2}}}{F_{\mathrm{startO_2}} - F_{\mathrm{endO_2}}} , \quad (4)$$

$$\dot{V}_{\mathrm{O_2}} = \left(\frac{V \times (F_{\mathrm{startO_2}} - F_{\mathrm{endO_2}})}{1 - (1 - \mathrm{RER}) \times F_{\mathrm{startO_2}}} \right) \div t , \quad (5)$$

$$\dot{V}_{\mathrm{CO2}} = \mathrm{RER} \times \dot{V}_{\mathrm{O2}} , \quad (6)$$

where RER is the respiratory gas exchange ratio, and F_{start} and F_{end} denote the fractions of CO_2 or O_2 determined at the start and end of the measurement. V is the volume of the respirometer chamber minus the volume of the water and the experimental animal (assuming a density of 1 ml g^{-1}), and t is the period during which the chamber was sealed. Metabolic rates were scaled to a 20 g animal using the formula:

$$\dot{V}_{\mathrm{O2(mass\ adjusted)}} = (20\,\mathrm{g} / \mathrm{animal\ mass})^{0.73} \times \dot{V}_{\mathrm{O2}} . \quad (7)$$

Statistical analysis

Mitochondrial oxygen consumptions were $\log_{10}$ transformed and Student's *t*-tests were performed to test for significant differences in respiration rates between control and aestivating animals. Student's *t*-tests were also used to test for significant differences in tissue oxygen consumption between control and aestivating animals. Whole animal oxygen consumption was $\log_{10}$ transformed and the data analysed using a one way ANOVA to test for significant differences between control and aestivator metabolic rate at the end of 10 weeks. All results are presented as means ± s.e.m. unless otherwise stated.

RESULTS

Mitochondrial metabolic rate

Mitochondrial respiration rates significantly decreased in aestivating frogs compared with controls. State 3 respiration rates decreased from 0.253±0.025 µl O_2 min^{-1} mg^{-1} protein in controls to 0.055±0.003 µl O_2 min^{-1} mg^{-1} protein in aestivators, equating to a 78% decrease in oxygen consumption (t=12.296, P<0.001; Fig. 1). State 4 respiration rates decreased by 88%, with a reduction from 0.085±0.011 µl O_2 min^{-1} mg^{-1} protein in controls to 0.010±0.002 µl O_2 min^{-1} mg^{-1} protein in aestivators (t=6.935, P<0.001; Fig. 1). The RCRs of the mitochondria significantly increased from 3.33±0.51 in controls to 8.1±2.62 in aestivators (t=–2.414, P=0.033; Fig. 2).

Tissue metabolic rate

The effect of aestivation on the oxygen consumption of tissue slices varied between the different tissues. Both the liver and gastrocnemius muscle showed significant reductions in oxygen consumption during aestivation (Fig. 3). Liver slices taken from aestivating animals showed a 53% reduction in oxygen consumption compared with control animals, decreasing from 86.2 ±14.2 µl O_2 h^{-1} g^{-1} tissue mass in controls to 40.4±4.8 µl O_2 h^{-1} g^{-1} tissue mass in aestivators (t=2.66, P=0.016; Fig. 3A). The oxygen consumption of the gastrocnemius was 34.4±1.6 and 24.1±1.2 µl O_2 h^{-1} g^{-1} tissue mass in controls and aestivators, respectively, a reduction in oxygen consumption of 30% (t=4.86, P<0.001; Fig. 3B).

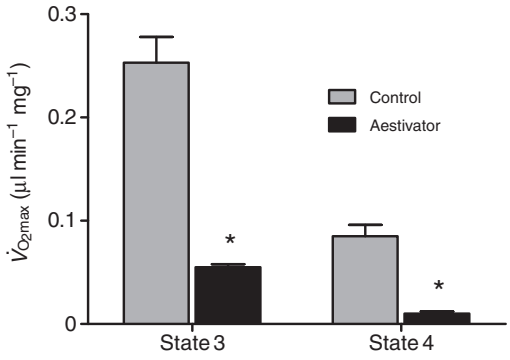

Fig. 1. Mitochondrial oxygen consumption (µl O_2 min^{-1} mg^{-1} protein, means ± s.e.m.) for respiration states 3 (ATP synthesis) and 4 (proton leak). Asterisks denote significant differences between treatment groups (P<0.001).

The mass of the liver in aestivating animals was significantly smaller than that in control animals (t=5.27, P<0.001; data not shown). As a result, when scaled to a 25 g animal, the oxygen consumption of the whole liver in aestivators was approximately 81% lower than that of controls, decreasing from 95.1±13.4 µl O_2 h^{-1} in controls to 17.9±2.7 µl O_2 h^{-1} in aestivators (t=8.18, P<0.001; Fig. 4).

Whole animal metabolic rate

After 10 weeks of aestivation, the metabolic rate of aestivating animals was significantly different from that of control animals, with mean oxygen consumptions of 7.9±1.9 and 43.0±6.6 µl O_2 h^{-1} g^{-1} body mass, respectively, equating to a decrease in oxygen consumption of 82% (t=4.48, P<0.001; Fig. 5). All aestivating animals had formed thin cocoons within 10 weeks of aestivation.

DISCUSSION

Mitochondria are the major contributors to total energy production during aerobic metabolism (Donohoe et al., 1998; Martin et al.,

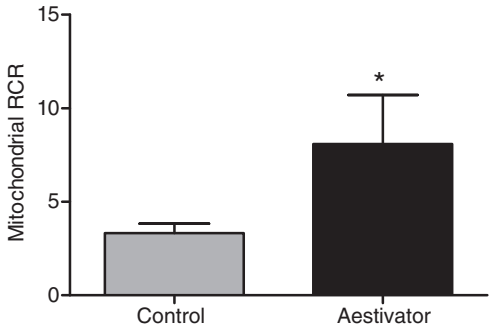

Fig. 2. Mitochondrial respiratory control ratios (RCR, means ± s.e.m.) for control and aestivating frogs. Asterisk denotes significant difference between treatment groups P=0.033).

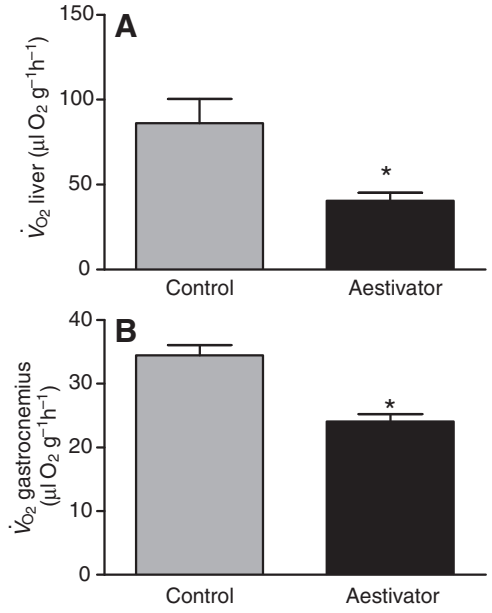

Fig. 3. Tissue metabolic rate (µl O_2 g^{-1} h^{-1}, means ± s.e.m.) of liver (A) and gastrocnemius (B). Asterisks denote significant differences between treatment groups (P<0.001).

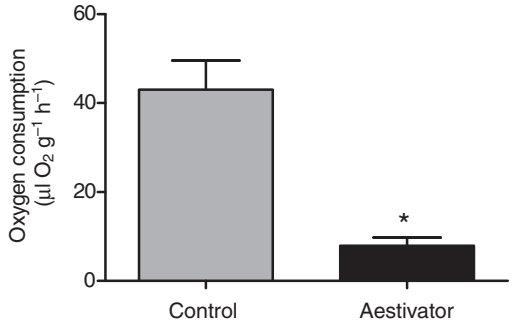

Fig. 5. Whole animal metabolic rate (µl O_2 g^{-1} h^{-1}, means ± s.e.m.) of control and 10 week aestivating frogs. Asterisk denotes significant difference between treatment groups (P<0.001).

1999) and as such are the likely site of any respiratory modifications associated with metabolic depression. After 7 months in aestivation, *C. alboguttata* had decreased mitochondrial oxygen consumption by an average of 83% and appeared to have increased mitochondrial coupling efficiency 2.5-fold. The significant energy savings observed at the mitochondrial level were reflected at higher levels of biological organisation, with individual organ oxygen consumption depressed by as much as 81% and whole animal metabolic rate by 82%.

Mitochondrial oxygen consumption is used to drive two processes: ATP synthesis and proton leak. The ratio of ATP synthesis to proton leak (respiratory control ratio or RCR) provides an indication of the degree of coupling of oxygen consumption to the synthesis of biologically usable energy (ATP synthesis) *versus* that 'wasted' in leak. In an animal that undergoes metabolic depression to conserve energy stores, it is reasonable to assume that both ATP production and proton leak would be down-regulated during the dormancy period, with proton leak down-regulated to a greater extent to maximise energy savings. This would be reflected by a higher RCR in dormant animals compared with active ones. In support of this hypothesis, after 7 months of aestivation the RCR for *C. alboguttata* had increased from 3.3 in active animals to 8.1 in aestivating frogs. This is the first known report of an increase in mitochondrial coupling efficiency in a dormant animal.

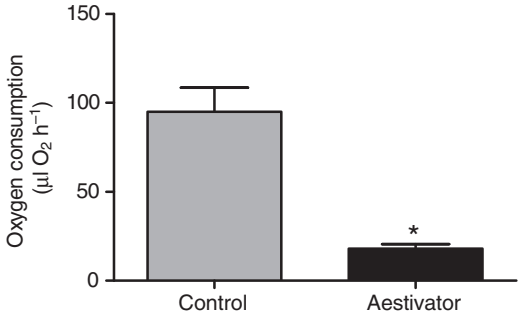

Fig. 4. Metabolic rate of whole liver (µl O_2 h^{-1}, means ± s.e.m.), scaled to a 25 g animal. Asterisk denotes significant difference between treatment groups (P<0.001).

Hibernating mammals show the reverse trend, i.e. they have reduced coupling efficiency compared with their active counterparts. During dormancy rates of ATP synthesis are decreased; however, proton leak rates remain at pre-hibernation levels (Liu et al., 1969; Pehowich and Wang, 1984; Gehnrich and Aprille, 1988; Brustovetsky et al., 1989; Brustovetsky et al., 1990; Brustovetsky et al., 1993; Martin et al., 1999). While this seems counterintuitive to the animal's need to conserve energy stores, it is likely that due to the endothermic nature of mammalian hibernators, proton leak is maintained to preserve a basal level of metabolism and heat production. Additional support for this hypothesis comes from the frog *Rana temporaria*, a hibernating ectotherm, and the snail *Helix aspersa*, an aestivating ectotherm, both of which have no need to maintain a relatively high body temperature during dormancy. Both *R. temporaria* and *H. aspersa* decreased both the rate of ATP synthesis and the rate of proton leak (Bishop and Brand, 2000; Bishop et al., 2002; Boutilier and St-Pierre, 2002). However, unlike in *C. alboguttata*, coupling efficiency in *R. temporaria* and *H. aspersa* did not increase; it was simply maintained by decreasing ATP synthesis and proton leak rates proportionately in relation to each other (Bishop and Brand, 2000; Bishop et al., 2002; Boutilier and St-Pierre, 2002).

This begs three questions. (1) How do *C. alboguttata* modulate proton leak. (2) If proton leak can be modulated, why do animals sustain proton leak at all. (3) What is it about *C. alboguttata* that allows them, unlike other dormant animals, to increase mitochondrial coupling efficiency during aestivation?

Two possible ways of increasing mitochondrial coupling efficiency are a reduction in membrane potential and/or changes to the 'leakiness' of the mitochondrial membrane (Boutilier and St-Pierre, 2002). A reduction in substrate utilisation during dormancy would result in decreased mitochondrial membrane potential (Bishop et al., 2002). As membrane potential drives both ATP synthesis and proton leak a decrease in membrane potential would result in decreased rates of ATP production and proton leak (Brand, 1990; Bishop and Brand, 2000; Bishop et al., 2002; Scheffler, 2008). The effect of membrane potential on ATP synthesis and proton leak is not linear and as such a decrease in membrane potential may have disproportionate effects on the rates of ATP synthesis and proton

leak, increasing coupling (Brand, 1990). The decrease in mitochondrial oxygen consumption observed in *R. temporaria* and *H. aspersa* is due to reduced substrate utilisation and thus reduced membrane potential; however, the effects on ATP synthesis and proton leak were proportional so the degree of coupling remained constant during dormancy (Bishop and Brand, 2000; Bishop et al., 2002; Boutilier and St-Pierre, 2002). Instead of, or in addition to, reduced substrate oxidation, changes to membrane leakiness may also occur. Such changes may include alterations in the fatty acid composition of the membrane and/or changes in the behaviour or number of protein uncouplers present within the membrane. There are several known proteins capable of catalysing mitochondrial uncoupling, including adenine nucleotide translocase (ANT), aspartate–glutamate carrier (AGC), uncoupling proteins (UCPs), and the proteins involved in the futile cycling of Ca^{2+} (Emel'yanova et al., 2003; Emel'yanova et al., 2004; Starkov, 2006; Harper et al., 2008). Although no data exist on changes in mitochondrial membrane fatty acid composition in aestivating frogs, Emel'yanova and colleagues (Emel'yanova et al., 2003) examined the uncoupling activity of ANTs and AGCs in the hibernating frog *R. temporaria* during winter (hibernation season) and spring. They reported a decrease in the uncoupling activity of AGCs in winter frogs compared with those measured in spring; however, there was no change in the activity of ANTs. The study does not report whether the winter animals were in hibernation at the time of measurement. Hudson and colleagues (Hudson et al., 2006) examined changes in gene expression of uncoupling protein 2 (UCP2) in the aestivating frog *C. alboguttata*. Uncoupling protein 1 (UCP1) is found exclusively in the brown adipose tissue (BAT) of mammals and when activated increases proton leak across the mitochondrial inner membrane, increasing heat production (Brand et al., 1999). UCP2 and UCP3 are homologues of UCP1 and, while not involved in thermogenesis, may be capable of catalysing mitochondrial proton leak in tissues other than BAT (St-Pierre et al., 2000) when in the presence of specific activators (Scheffler, 2008). Hudson and colleagues (Hudson et al., 2006) reported a non-signifcant trend for down-regulation of UCP2 transcripts in 6 month aestivating *C. alboguttata* skeletal muscle; changes in protein expression were not examined.

The reduction in the production of damaging reactive oxygen species (ROS) appears to be a key role of proton leak (Rolfe and Brown, 1997; Boutilier and St-Pierre, 2002; Duval et al., 2002). UCP2/3 are activated in response to the presence of ROS and probably play a significant role in the protection against ROS damage (Scheffler, 2008). A lowered aerobic metabolism during dormancy would lead to a reduction in ROS production (Grundy and Storey, 1998), reducing the need for UCP2/3 expression, and may result in decreased rates of proton leak and increased mitochondrial coupling during dormancy. However, oxidative damage may increase during arousal due to a phenomenon called reperfusion stress (Staples and Brown, 2008). The relative maintenance of high UCP2 mRNA levels in *C. alboguttata* during aestivation (Hudson et al., 2006) may be a pre-emptive measure that allows immediate uncoupling of mitochondria to combat potential ROS damage upon awakening by providing a readily available pool of transcripts.

The massive energy savings recorded in this study at the mitochondrial level were also reflected at both the tissue and whole animal level. Within 10 weeks of aestivation whole animal metabolic rate had decreased by 82%. After 7 months of aestivation significant decreases in metabolic rate were observed in the gastrocnemius muscle and the liver of *C. alboguttata*. Oxygen consumption of the gastrocnemius muscle decreased by 30% during aestivation. Skeletal muscle represents approximately 35% of a frog's body mass (Putnam, 1979), and as such metabolic depression in muscle will play an important role in whole animal metabolic depression. The oxygen consumption of the liver in *C. alboguttata* decreased by 53% in aestivating animals compared with controls. We also observed a significant decrease in the size of the liver of *C. alboguttata* during aestivation. The decrease in oxygen consumption per gram of tissue, coupled with the decrease in the size of the liver, resulted in an overall decrease in liver energy expenditure of 81%.

Concluding remarks

In an animal that must survive extended periods in the absence of food and water, maximising energy savings during metabolic depression is a necessity, especially when the duration of metabolic depression is unpredictable. The green-striped burrowing frog, *C. alboguttata*, shows a remarkable ability to depress metabolism during aestivation at all levels of biological organisation. An interesting finding of this study is the potential increase in mitochondrial coupling during dormancy as reflected by an increase in RCR values. An increased mitochondrial RCR has previously been shown to be associated with an increase in whole animal energy efficiency (Bottje et al., 2002; Kolath et al., 2006; Bo et al., 2008; Andreu et al., 2009). From our findings we propose the hypothesis that dormant *C. alboguttata* become more energy efficient, which may help to explain the superior lengths of dormancy documented in these frogs compared with hibernators.

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