

## RESEARCH ARTICLE

# Decreased hydrogen peroxide production and mitochondrial respiration in skeletal muscle but not cardiac muscle of the green-striped burrowing frog, a natural model of muscle disuse

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**ABSTRACT**

Suppression of disuse-induced muscle atrophy has been associated with altered mitochondrial reactive oxygen species (ROS) production in mammals. However, despite extended hindlimb immobility, aestivating animals exhibit little skeletal muscle atrophy compared with artificially immobilised mammalian models. Therefore, we studied mitochondrial respiration and ROS ( $H_2O_2$ ) production in permeabilised muscle fibres of the green-striped burrowing frog, *Cyclorana alboguttata*. Mitochondrial respiration within saponin-permeabilised skeletal and cardiac muscle fibres was measured concurrently with ROS production using high-resolution respirometry coupled to custom-made fluorimeters. After 4 months of aestivation, *C. alboguttata* had significantly depressed whole-body metabolism by ~70% relative to control (active) frogs, and mitochondrial respiration in saponin-permeabilised skeletal muscle fibres decreased by almost 50% both in the absence of ADP and during oxidative phosphorylation. Mitochondrial ROS production showed up to an 88% depression in aestivating skeletal muscle when malate, succinate and pyruvate were present at concentrations likely to reflect those *in vivo*. The percentage ROS released per  $O_2$  molecule consumed was also ~94% less at these concentrations, indicating an intrinsic difference in ROS production capacities during aestivation. We also examined mitochondrial respiration and ROS production in permeabilised cardiac muscle fibres and found that aestivating frogs maintained respiratory flux and ROS production at control levels. These results show that aestivating *C. alboguttata* has the capacity to independently regulate mitochondrial function in skeletal and cardiac muscles. Furthermore, this work indicates that ROS production can be suppressed in the disused skeletal muscle of aestivating frogs, which may in turn protect against potential oxidative damage and preserve skeletal muscle structure during aestivation and following arousal.

**KEY WORDS:** *Cyclorana alboguttata*, Mitochondria, Muscle atrophy, Oxidative stress, Reactive oxygen species, Skeletal muscle

**INTRODUCTION**

Aestivation is a state of dormancy that enables numerous animals (invertebrates, fish, frogs and reptiles) to survive under desiccating conditions for extended periods of time. In arid and semi-arid environments where food and water are often limiting, aestivating

animals promote survival by coordinately suppressing a suite of physiological and biochemical processes (e.g. hypophagia, hypoventilation, hypometabolism), decreasing locomotor activity, and persisting solely on endogenous energy stores (Storey and Storey, 1990). Green-striped burrowing frogs (*Cyclorana alboguttata* Günther 1867) survive in drought-affected areas of Australia by burrowing underground, shedding a waterproof cocoon, and aestivating for extended periods (months to years). Although the cocoon limits evaporative water loss, it also secondarily hinders skeletal muscle movement as the hindlimbs are completely immobilised. Despite this prolonged muscle inactivity, skeletal muscle atrophy has been shown to be minimal and muscle functional capacity maintained in frogs following aestivation (Hudson and Franklin, 2002a; Symonds et al., 2007; Mantle et al., 2009). *Cyclorana alboguttata* aestivating for 9 months show no loss in myofibre cross-sectional area (CSA; a marker of muscle atrophy) in the gastrocnemius, an important muscle that produces power necessary for jumping (Mantle et al., 2009). In contrast, hindlimb immobilisation in conventional experimental models, such as rats, can result in a significant (up to 32%) loss in gastrocnemius myofibre CSA in as little as 2 weeks (Sakakima et al., 2004). Disuse-induced skeletal muscle atrophy has been linked to increased reactive oxygen species (ROS) production in muscle fibres, leading to oxidative stress and muscle tissue damage (Powers et al., 2011). For example, prolonged bed rest in humans can result in increased carbonylation of muscle proteins and an apparent weakening of antioxidant defence systems (Dalla Libera et al., 2009; Brocca et al., 2012).

ROS are formed as by-products of normal aerobic cellular metabolism. A number of comparative studies suggest that species with higher mass-specific metabolic rates have elevated ROS production (Adelman et al., 1988; Lopez-Torres et al., 1993; Foksinski et al., 2004). Consequently, it has been hypothesised that metabolic suppression during dormancy leads to a decrease in ROS production in muscle fibres, which may be a potential means of reducing the effects of muscle disuse atrophy in natural models of muscle disuse (i.e. aestivating frogs and hibernating mammals) (Hudson and Franklin, 2002b). While skeletal muscle is effectively dormant throughout aestivation (Kayes et al., 2009a; Kayes et al., 2009b), cardiac muscle must remain active to ensure adequate perfusion of organs. This is particularly true of cocoon-forming aestivating frogs, which have an increased reliance on pulmonary gas exchange and pulmonary circulation relative to non-cocoon-forming species (Loveridge and Withers, 1981). In the mammalian heart, ROS are an important determinant of cardiomyocyte homeostasis and proper contractile function. Whereas low concentrations can stimulate signal transduction processes, high concentrations may lead to cardiomyocyte injury (Suzuki and Ford, 1999; Seddon et al., 2007). Little is understood about ROS production and signalling during metabolic depression; therefore, it

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is of interest to explore ROS in distinct tissues that respond differently throughout the dormant phase.

Oxidative stress occurs only when ROS overwhelm the detoxifying capacity of cells. One way in which cells can protect themselves from potentially lethal oxidative damage is to increase the synthesis and/or activity of intracellular antioxidant enzymes. Numerous studies have demonstrated the induction of antioxidant defences during dormancy (including aestivation), suggesting that enhanced oxidative stress resistance is an integral component of metabolic suppression (for reviews, see Carey et al., 2003; Ferreira-Cravo et al., 2010). In *C. alboguttata*, aestivation for 4 months resulted in the induction of mRNA transcripts associated with skeletal muscle nuclear factor erythroid 2-related factor 2 (Nrf2), a regulator of the oxidative stress response (Reilly et al., 2013). Furthermore, water-soluble and membrane-bound antioxidants and gene expression levels of muscle catalase and glutathione peroxidase were shown to be maintained at control levels in muscles of dormant frogs (Hudson et al., 2006). These studies indicate that modulation of antioxidants in aestivating muscle might decrease the susceptibility of muscle fibres to the atrophic effects of oxidative stress. As direct measurements of ROS are complicated and often exposed to errors, redox balance during dormancy has been typically studied indirectly by examination of lipid peroxidation and/or protein carbonylation (Grundy and Storey, 1998; Young et al., 2013). In one of the few studies that measured ROS production directly, there was generally no difference observed in mitochondrial ROS production in skeletal muscle of dormant versus interbout euthermic ground squirrels (Brown et al., 2012). However, this is perhaps unsurprising as measurements are typically conducted at saturating substrate concentrations (e.g. 5–10 mmol l<sup>-1</sup> succinate), whereas substrate inputs to mitochondria are probably substantially depressed *in vivo*. Moreover, substrates used in assays are often chosen to maximise net ROS production and succinate (Bishop and Brand, 2000; St-Pierre et al., 2000; Armstrong and Staples, 2010; Gallagher and Staples, 2013), whereas *in vivo* substrates will be a composite of electron inputs to complexes I and II.

In the current study we examined mitochondrial respiration and ROS production within permeabilised cardiac and skeletal muscle fibres of 4 month aestivating *C. alboguttata*. A major aim in the present study was to add mitochondrial substrates together in proportions that are likely to reflect substrates present *in vivo*, and to understand the response of mitochondria in the aestivating condition when substrate supply and oxidation should be suppressed. Furthermore, we aimed to answer the following questions. (1) Are ROS produced at a lower rate in skeletal and cardiac muscle of aestivating *C. alboguttata* compared with active awake animals? (2) How does substrate concentration reflect electron inputs and ROS leakage in different physiological states? We hypothesised that mitochondrial respiration in skeletal and cardiac muscle would be suppressed in aestivating *C. alboguttata*, and that they would generate less ROS during distinct respiratory states (e.g. without adenylates versus during ATP production) with substrate inputs that reflect depressed blood glucose. To test our hypothesis, we used high resolution respirometry in conjunction with custom-made fluorometers that concurrently measured mitochondrial respiration and ROS production in permeabilised cardiac and skeletal muscle fibres.

## RESULTS

### Muscle mass and blood glucose

There was no significant difference in body size (snout–vent length, SVL) of aestivating *C. alboguttata* (mean ± s.e.m. 6.13±0.93 cm)

compared with controls (6.21±1.32 cm;  $P=0.66$ ). Four months of aestivation resulted in an 8% reduction in the wet mass of gastrocnemius muscle of aestivating *C. alboguttata* (359.59±17.35 mg) relative to control frogs (390.03±21.34 mg). The effect of aestivation on gastrocnemius wet mass was not significant when the SVL of frogs was accounted for (ANCOVA: full model,  $P=0.69$ ; treatment,  $P=0.34$ ; relationship to SVL,  $P\leq 0.05$ ). The blood glucose of aestivating frogs (0.96±0.06 mmol l<sup>-1</sup>) was significantly lower than that of control frogs (1.66±0.08 mmol l<sup>-1</sup>;  $P\leq 0.001$ ).

### Whole-animal metabolic rate and muscle mitochondrial respiration

The whole-animal O<sub>2</sub> consumption of *C. alboguttata* decreased by ~70%, from 66.2±7.5 μl O<sub>2</sub> g<sup>-1</sup> h<sup>-1</sup> for control individuals to 20.3±3.2 μl O<sub>2</sub> g<sup>-1</sup> h<sup>-1</sup> for individuals after 4 months of aestivation ( $P\leq 0.001$ ; Fig. 1). Given that substrates used to measure mitochondrial respiration were mixed, we simplified the substrate concentrations to their respective electron inputs assuming that pyruvate (*in vitro*) oxidation results in 3 NADH + H<sup>+</sup>, malate 1 NADH + H<sup>+</sup>, and succinate 1 FADH<sub>2</sub>. Multiplication of each substrate's concentration by the electron contribution and then by Avogadro's constant (6.02×10<sup>23</sup>) provides an approximation of the number of electrons that can be donated to the electron transport system. Mitochondrial respiratory flux rates in both skeletal and cardiac muscles obeyed typical Michaelis–Menten kinetics with increasing substrate concentrations (i.e. LEAK<sub>N</sub>; respiration without adenylates present; Fig. 2A,C). In skeletal muscle the maximal respiratory flux ( $V_{\max}$ ) decreased from 3.10±0.10 pmol O<sub>2</sub> s<sup>-1</sup> mg<sup>-1</sup> wet mass in controls to 1.79±0.15 pmol O<sub>2</sub> s<sup>-1</sup> mg<sup>-1</sup> wet mass in aestivators, equating to a 42% decrease in oxygen consumption ( $P\leq 0.0001$ ; Table 1). By contrast, the apparent  $K_m$  (the substrate concentrations at which respiratory flux was half of  $V_{\max}$ ) was not significantly different between control and aestivating frogs ( $P=0.80$ ; Table 1). Respiratory flux in skeletal muscle fibres during oxidative phosphorylation (OXPHOS) decreased by 46%, from 9.86±1.31 pmol O<sub>2</sub> s<sup>-1</sup> mg<sup>-1</sup> wet mass in controls to 5.31±0.94 pmol O<sub>2</sub> s<sup>-1</sup> mg<sup>-1</sup> wet mass in aestivating frogs ( $P\leq 0.05$ , Fig. 2B). Both the  $V_{\max}$  and  $K_m$  of aestivating cardiac muscle were maintained at levels similar to that of control animals during LEAK<sub>N</sub> ( $P=0.16$  and 0.33,

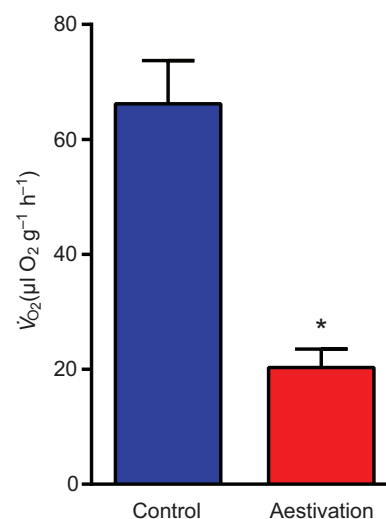
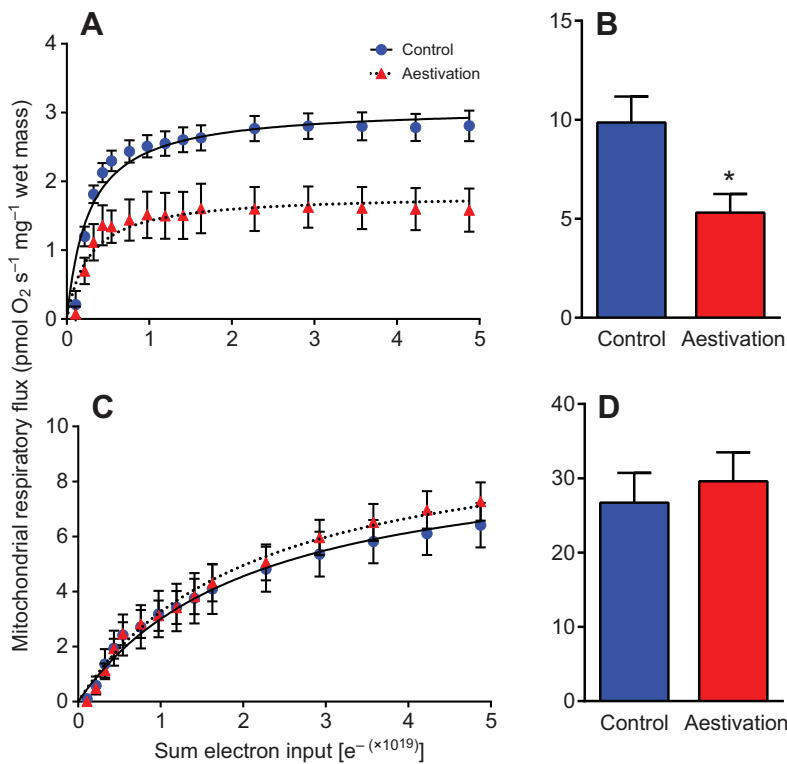


Fig. 1. Whole-animal oxygen consumption ( $\dot{V}O_2$ ) of *Cyclorana alboguttata* at rest (control,  $N=10$ ) and after 4 months of aestivation ( $N=12$ ). Data were analysed using one-way ANOVA and are presented as means ± s.e.m., \* $P<0.001$ .



**Fig. 2. Mitochondrial respiratory flux in permeabilised skeletal and cardiac muscle fibres of *C. alboguttata*.** (A,B) Skeletal muscle: control, *N*=5; aestivation, *N*=6; (C,D) cardiac muscle: control, *N*=5; aestivation, *N*=7. Respiratory flux in muscle is shown in the presence of the mitochondrial substrates malate, succinate and pyruvate only (LEAK<sub>N</sub>; A,C), and the rate of respiratory flux in muscle is shown during maximum oxidative phosphorylation (OXPHOS; B,D). In A and C, the x-axis is presented as an approximation of the number of electrons that can be donated by malate, succinate and pyruvate to the electron transport system. *V*<sub>max</sub> and *K*<sub>m</sub> values during LEAK<sub>N</sub> were compared between 4 month aestivating and control frogs and tested for significance using an extra sum-of-squares *F*-test. Mitochondrial respiration during OXPHOS was analysed by one-way ANOVA. Data are means ± s.e.m., \**P*<0.05.

respectively; Table 1). Similarly, mitochondrial respiratory flux in the heart during OXPHOS was unaffected by aestivation (*P*=0.63; Fig. 2D).

**Hydrogen peroxide production in permeabilised cardiac and skeletal muscle fibres**

In skeletal and cardiac muscle hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) production could not be distinguished from background levels until the fifth cocktail injection, when cumulative substrate concentrations of pyruvate, succinate and malate were 0.5, 0.5 and 0.25 mmol l<sup>-1</sup>, respectively. Because H<sub>2</sub>O<sub>2</sub> production did not strictly follow a Michaelis–Menten model during LEAK<sub>N</sub>, we determined whether H<sub>2</sub>O<sub>2</sub> production differed between control and aestivating frogs at individual substrate concentrations. At low cumulative substrate concentrations, H<sub>2</sub>O<sub>2</sub> production was significantly lower in skeletal muscle of aestivating frogs (Fig. 3A, *P*=0.04 and *P*=0.02, respectively), as was the amount of H<sub>2</sub>O<sub>2</sub> formed per O<sub>2</sub> (an indication of the %ROS of O<sub>2</sub>; Fig. 4A, *P*=0.04). H<sub>2</sub>O<sub>2</sub> production was ~12% that of control frogs when cumulative substrate concentrations of pyruvate, succinate and malate were 1.3, 1.3 and 0.65 mmol l<sup>-1</sup>, respectively, while H<sub>2</sub>O<sub>2</sub> formed per O<sub>2</sub> was ~6% that of control frogs at similar concentrations. However, as mitochondrial

substrate concentrations were increased in the medium, H<sub>2</sub>O<sub>2</sub> production did not differ significantly between aestivators and controls, because of a large variation among control animals. Both H<sub>2</sub>O<sub>2</sub> production and %ROS of O<sub>2</sub> from aestivating cardiac muscle fibres were similar to that of controls all throughout LEAK<sub>N</sub>. During OXPHOS, H<sub>2</sub>O<sub>2</sub> production and %ROS of O<sub>2</sub> in skeletal and cardiac muscle were not significantly different between aestivators and controls (H<sub>2</sub>O<sub>2</sub> production, *P*=0.18 and *P*=0.76, respectively; %ROS of O<sub>2</sub>, *P*=0.07 and *P*=0.49, respectively). In general, ROS production was more tightly regulated (i.e. less variable) in aestivating frogs than in control animals.

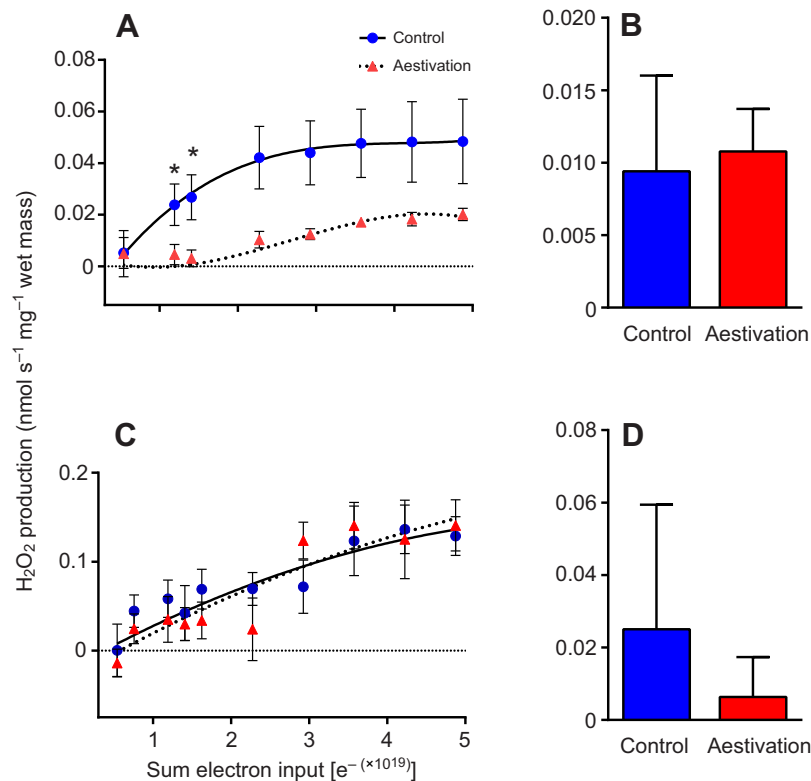
**DISCUSSION**

In humans and most other mammals, prolonged skeletal muscle disuse leads to a loss of muscle protein and fibre atrophy. It has been shown that long periods of limb immobilisation stimulate increased ROS production in disused fibres (Min et al., 2011). Though many studies have investigated muscle antioxidant levels during dormancy and/or arousal in aestivators and hibernators (Ramos-Vasconcelos and Hermes-Lima, 2003; Hudson et al., 2006; Allan and Storey, 2012; James et al., 2013; Young et al., 2013), little is known about changes in mitochondrial ROS production and this has only recently received experimental attention in natural models of muscle disuse (Brown et al., 2012). Additionally, relatively few physiological studies examine mitochondrial function using permeabilised fibres, tissues or cells. In the current study, we have verified the use of saponin-permeabilised muscle fibres (Kuznetsov et al., 2008), an approach that is more likely to resemble conditions in living cells than analyses using isolated mitochondria preparations. We have shown that aestivating *C. alboguttata* are capable of selectively suppressing or maintaining rates of mitochondrial respiration within distinct muscle tissue types and our study is the first to measure net mitochondrial ROS production (i.e. the sum of H<sub>2</sub>O<sub>2</sub> production that escapes the mitochondrial antioxidant system)

**Table 1. Mitochondrial respiratory flux followed a Michaelis–Menten model in permeabilised skeletal and cardiac muscle fibres of *Cyclorana alboguttata***

	<i>V</i> <sub>max</sub> (pmol O <sub>2</sub> s <sup>-1</sup> mg <sup>-1</sup> )	<i>K</i> <sub>m</sub> [e <sup>-</sup> (×10 <sup>19</sup> )]
Skeletal muscle		
Control ( <i>N</i> =5)	3.10±0.10	0.28±0.04
Aestivation ( <i>N</i> =6)	1.79±0.15***	0.25±0.10
Heart		
Control ( <i>N</i> =5)	8.57±1.11	1.71±0.51
Aestivation ( <i>N</i> =7)	10.89±1.19	2.44±0.54

\*\*\**P*<0.0001.

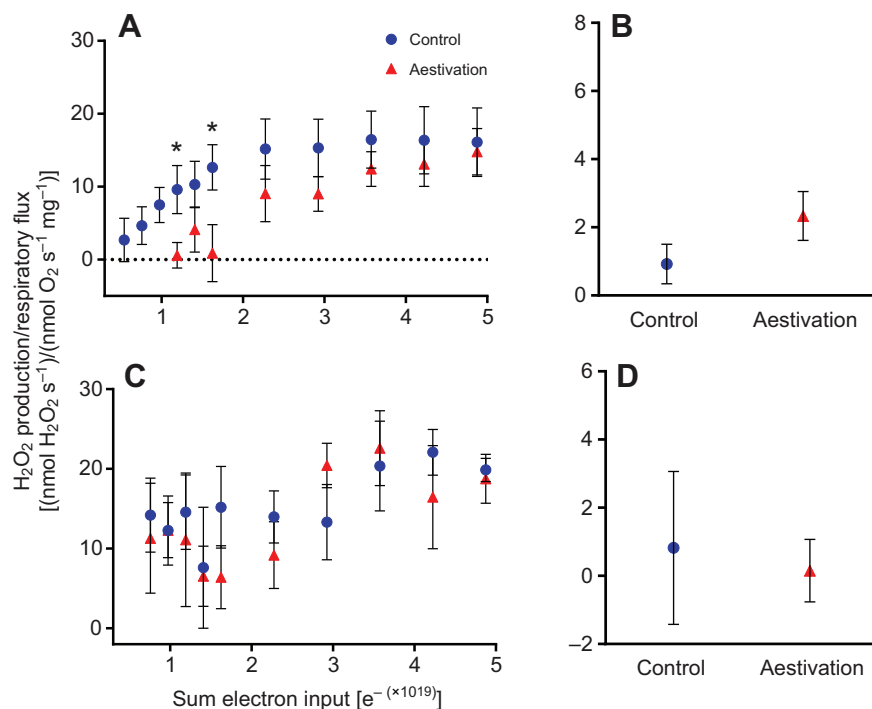


**Fig. 3. Hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) production in permeabilised skeletal and cardiac muscle fibres of *C. alboguttata*.** (A,B) Skeletal muscle: control,  $N=5$ ; aestivation,  $N=6$ ; (C,D) cardiac muscle: control,  $N=5$ ; aestivation,  $N=7$ .  $\text{H}_2\text{O}_2$  production was achieved by the addition of superoxide dismutase to the oxygraph chamber and determined in different respiration states ( $\text{LEAK}_N$ , A, C; and OXPHOS, B,D) as outlined in Materials and methods. In skeletal muscle,  $\text{H}_2\text{O}_2$  production tended to be lower in aestivating animals during  $\text{LEAK}_N$ , whereas in cardiac muscle,  $\text{H}_2\text{O}_2$  production remained similar in control and aestivating frogs. There was no significant difference in skeletal or heart muscle  $\text{H}_2\text{O}_2$  production between controls and aestivators during OXPHOS. In A and C the x-axis is presented as an approximation of the number of electrons that can be donated by malate, succinate and pyruvate to the electron transport system. During  $\text{LEAK}_N$ ,  $\text{H}_2\text{O}_2$  production was tested for significance using individual  $t$ -tests at each separate mitochondrial substrate injection point, whereas  $\text{H}_2\text{O}_2$  production during OXPHOS was analysed using a Wilcoxon rank sum test. Data are means  $\pm$  s.e.m.,  $*P<0.05$ .

during aestivation using a combination of mitochondrial substrates, which better reflects physiological conditions. We have also demonstrated that *C. alboguttata* are able to suppress ROS production in disused skeletal muscle at low substrate concentrations. Unlike in skeletal muscle, ROS production in permeabilised cardiac muscle fibres appeared unaffected by aestivation. Overall, the current study enhances our understanding of the control of mitochondrial respiration and ROS production in aestivating animals.

#### Mitochondrial respiration

Mitochondria are the principal sites of skeletal muscle fuel metabolism and ATP production. It follows then that mitochondrial metabolism should be suppressed in disused skeletal muscles of aestivating or hibernating animals. Following 4 months of aestivation, *C. alboguttata* had depressed skeletal muscle mitochondrial respiratory flux by  $\sim 45\%$  and whole-animal metabolic rate by almost 70%. The suppression of skeletal muscle mitochondrial and whole-animal respiration clearly maximises



**Fig. 4.  $\text{H}_2\text{O}_2$  production/mitochondrial respiratory flux in permeabilised skeletal and cardiac muscle fibres of *C. alboguttata*.** (A,B) Skeletal muscle: control,  $N=5$ ; aestivation,  $N=6$ ; (C,D) cardiac muscle: control,  $N=5$ ; aestivation,  $N=7$ .  $\text{H}_2\text{O}_2$  production was divided by mitochondrial respiratory flux in different respiration states ( $\text{LEAK}_N$ , A, C; and OXPHOS, B,D) to provide an indication of  $\text{H}_2\text{O}_2$  produced per  $\text{O}_2$  turned over (%ROS of  $\text{O}_2$ ). In skeletal muscle, %ROS of  $\text{O}_2$  was significantly lower in aestivating animals during  $\text{LEAK}_N$  at low substrate concentrations, whereas in cardiac muscle, %ROS of  $\text{O}_2$  remained similar in control and aestivating frogs. There was no significant difference in skeletal or heart muscle %ROS of  $\text{O}_2$  between controls and aestivators during OXPHOS. In A and C the x-axis is presented as an approximation of the number of electrons that can be donated by malate, succinate and pyruvate to the electron transport system. Individual  $t$ -tests were used to assess significance. Data are means  $\pm$  s.e.m.,  $*P<0.05$ .



energy savings for aestivating frogs. These results are in agreement with previous work on *C. alboguttata*, which demonstrated suppression of skeletal muscle mitochondrial and whole-animal respiration during aestivation by more than 80% (Kayes et al., 2009b). The greater magnitude of metabolic depression in that study may be related to a longer period of aestivation and/or differences in the preparation of isolated mitochondria.

Whereas both resting and active mitochondrial respiration were significantly depressed in skeletal muscle of aestivators, cardiac muscle mitochondrial respiration remained similar between aestivating and control frogs across all respiratory states. In amphibians, the response of the heart during aestivation varies depending on the species. Heart rate has been shown to decrease (Gehlbach et al., 1973; Seymour, 1973; Glass et al., 1997) or remain unchanged (Loveridge and Withers, 1981) in aestivators when compared with their awake conspecifics. While the coordinated downregulation of many organ and cell functions is a key priority during dormancy (e.g. transport across cell membranes, transcription, protein synthesis), aestivators must also reprioritise the use of ATP to support critical functions. The maintenance of mitochondrial respiration in aestivating *C. alboguttata* cardiac muscle at control levels suggests that aestivators continue to produce ATP in the heart for important functions such as contraction and relaxation, and membrane transport systems (e.g.  $\text{Na}^+/\text{K}^+$ -ATPase). This is consistent with the requirement to continue adequate delivery of blood and oxygen to the tissues, whilst ensuring the cardiovascular system is ready to sustain sudden activity upon arousal from aestivation. Our heart data are supported by recent studies examining mitochondrial respiration of cardiac muscles in hamsters (*Phodopus sungorus*) and squirrels (*Ictidomys tridecemlineatus*) (Gallagher and Staples, 2013; Kutschke et al., 2013). In both these studies, torpid animals were shown to maintain their rate of cardiac mitochondrial oxygen consumption at levels similar to that of control (i.e. interbout euthermic) animals across a range of respiratory states.

### ROS ( $\text{H}_2\text{O}_2$ ) production

In the current study we hypothesised that aestivating *C. alboguttata* would produce less ROS from permeabilised muscle fibres relative to awake frogs. Skeletal muscle ROS production during LEAK<sub>N</sub> tended to be lower in aestivating animals, and was significantly decreased at sub-saturating substrate concentrations. Furthermore, aestivating frogs also produced less ROS per  $\text{O}_2$  turned over, which suggests that aestivators can modulate the handling of electrons in the electron transport system independently of simply suppressing electron flow. We note there was particularly high variation in ROS production among control frogs at higher, saturating substrate concentrations, precluding a statistically significant difference between aestivators and controls in this latter part of the experiment. In a recent study, Brown et al. (Brown et al., 2013) suggested that sub-saturating mitochondrial substrate (succinate) concentrations are more physiologically relevant *in vivo*. Indeed, the concentration of succinate in many mammalian tissues is considered to be low, in the  $0.2\text{--}0.5\text{ mmol l}^{-1}$  range (Starkov, 2008). Succinate (or malate or pyruvate) concentration data are not presented as these metabolites change rapidly (Zoccarato et al., 2009). However, blood glucose concentrations were much lower in aestivators than in active *C. alboguttata* (present study) and aestivators are likely to have a decreased reliance on carbohydrate metabolism in skeletal muscle (Storey and Storey, 2010; Reilly et al., 2013). While lipid-based substrates may dominate carbohydrates in aestivating animals, fatty acids can uncouple mitochondria, further suppressing ROS production in aestivators.

Previous studies have shown that aestivating *C. alboguttata* sustains hindlimb muscle mass until 6–9 months of aestivation (Hudson et al., 2006; Mantle et al., 2009). We suggest that decreased ROS production in 4 month aestivating skeletal muscle may represent a mechanism by which dormant *C. alboguttata* limit muscle fibre atrophy. Indeed, a recent study found no evidence of lipid or protein oxidation (indices of ROS-induced oxidative damage) in the gastrocnemius muscle of *C. alboguttata* following 6 months aestivation (Young et al., 2013). Previous studies have also emphasised the protective effects of increased muscle antioxidant production in dormant burrowing frogs (Hudson and Franklin, 2002b; Hudson et al., 2006; Reilly et al., 2013). Together, these experimental data suggest that *C. alboguttata* maintain an appropriate ratio of antioxidants to pro-oxidants, and this should prevent oxidative stress and premature skeletal muscle fibre atrophy. Decreased production of ROS in *C. alboguttata* skeletal muscle is in contrast to what has been observed during immobilisation-induced muscle atrophy in mammalian models (Min et al., 2011). Two weeks of cast immobilisation in mice resulted in both increased rates of mitochondrial  $\text{H}_2\text{O}_2$  release from permeabilised skeletal muscle fibres and higher levels of muscle lipid peroxidation, while administration of a mitochondria-targeted antioxidant to mice also inhibited the increase in muscle mitochondrial  $\text{H}_2\text{O}_2$  production and attenuated myofibre atrophy.

Given that mitochondrial respiration in *C. alboguttata* permeabilised cardiac muscle fibres was not different between controls and aestivators, it is perhaps not surprising that mitochondrial ROS production from the heart was also unchanged. Because of its high energetic demand and abundance of mitochondria, the heart is presumably very sensitive to oxidative damage. Data on the production of mitochondrial ROS from heart tissue and their role in cell signalling during dormancy are lacking in the literature. However, there is little evidence for oxidative damage occurring in cardiac muscle during aestivation, while protein and enzyme activity levels of antioxidants within the heart have been shown to increase, decrease or remain unchanged depending on the species, duration of aestivation and specific antioxidant measured (Grundy and Storey, 1998; Page et al., 2010; Salway et al., 2010). It is difficult to draw conclusions about the effects of ROS production in aestivating *C. alboguttata* cardiac muscle. It is conceivable that aestivating *C. alboguttata* may modulate antioxidants in the heart to protect macromolecules from potentially lethal stress-induced damage. However, ROS have been shown to significantly contribute as regulators of cell signalling pathways in model organisms (Burgoyne et al., 2012), and ROS are likely to have similar roles in cardiomyocytes of other vertebrates as well. Clearly, additional well-designed experiments are needed to determine the relative importance of ROS in cell signalling and/or oxidative stress in heart tissue during dormancy.

### Concluding remarks

We have shown that *C. alboguttata* heart and skeletal muscle tissue respond differently during aestivation with respect to mitochondrial respiration and ROS production. This is of particular interest, as it exemplifies *C. alboguttata*'s capacity to independently regulate distinct organs throughout dormancy. The downregulation of mitochondrial respiration in gastrocnemius muscle is consistent with markedly reduced muscle contraction throughout the aestivating period, allowing significant energy savings for dormant frogs. Muscle is a highly excitable tissue and its metabolic rate can increase rapidly within a very brief period of time. Thus, it is likely that skeletal muscle mitochondrial respiration is quickly restored to normal levels to facilitate muscle contraction when aestivating *C.*

*alboguttata* arouse. Whereas skeletal muscle essentially ceases function but can contract upon arousal, it is imperative that the burrowing frog heart maintains its morphology and contractile activity during aestivation. Maintenance of mitochondrial respiration in *C. alboguttata* cardiac muscle would allow the slow but sustained supply of ATP for critical heart functions. ROS production generally reflected mitochondrial respiration in the different muscles. At low mitochondrial substrate concentrations, ROS production was significantly lower in the gastrocnemius muscle fibres of aestivating burrowing frogs, which may represent a mechanism contributing to the limited muscle atrophy observed in this species despite extended hindlimb disuse. Production of ROS in cardiac muscle fibres did not change during aestivation, and further research is required to determine the roles of mitochondrial ROS in cardiomyocyte signalling and homeostasis during metabolic depression.

## MATERIALS AND METHODS

### Experimental animals and whole-animal metabolic rate

The experiments were approved by the University of Queensland Animal Ethics Committee (approval no. SBS/238/11/ARC). Green-striped burrowing frogs (*C. alboguttata*) were collected after summer rainfall from roadsides in the Darling Downs region of Queensland, Australia, under Scientific Purposes Permit WISP10060511. Frogs were housed in the laboratory in individual plastic boxes containing wet paper towelling and were provided with water and fed live crickets *ad libitum*. Frogs were allocated to their treatment groups (controls or 4 month aestivators), with treatments matched as closely as possible for body mass and sex. To induce aestivation, frogs were placed into individual 500 ml glass chambers filled with wet paper pellets that were allowed to dry out naturally over a period of several weeks. Animals burrowed into the paper pellets as the chambers dried out and adopted a water-conserving posture. All frogs were maintained in a temperature-controlled room (23°C) with a 12 h:12 h light/dark regime. Aestivating frogs were kept in cardboard boxes to reduce the effects of light disturbance. Throughout the experiment, whole-animal metabolism was measured in aestivating ( $N=12$ ) and control frogs ( $N=10$ ) as previously described (Reilly et al., 2013). Briefly, aestivators remained in their chambers for the entire experimental period whereas control animals were weighed and placed into their chambers 24 h prior to sampling and removed immediately following final oxygen consumption measurements. Control frogs were then fed. Rates of oxygen consumption ( $\dot{V}_{O_2}$ ) were measured using closed-system respirometry using a fibre optic oxygen transmitter with oxygen-sensitive spots (Precision Sensing GmbH, Regensburg, Germany), which measure the partial pressure of oxygen (as a percentage of air saturation) within the chamber. Oxygen measurements were taken several hours later, depending on the treatment group (i.e. longer for aestivators), and on multiple occasions to calculate repeated rates of oxygen consumption. After 4 months, all aestivating animals had formed thin cocoons around their bodies.

### Preparation of permeabilised muscle fibres

The permeabilised skeletal and cardiac muscle fibre preparations were performed following previous methods (Hickey et al., 2012), which avoids problems associated with traditional mitochondrial isolation methods (Picard et al., 2011). All frogs were killed by cranial and spinal pithing. Immediately following pithing, blood glucose of individual frogs was measured using an Accu-Chek Performa Blood Glucose Meter and test strips (Roche, Castle Hill, NSW, Australia). Both the heart and the left gastrocnemius muscle were dissected, weighed and placed immediately into ice-cold muscle relaxant buffer containing 10 mmol l<sup>-1</sup> Ca-EGTA buffer, 20 mmol l<sup>-1</sup> imidazole, 20 mmol l<sup>-1</sup> taurine, 50 mmol l<sup>-1</sup> K-MES, 0.5 mmol l<sup>-1</sup> DTT, 6.56 mmol l<sup>-1</sup> MgCl<sub>2</sub>, 5.77 mmol l<sup>-1</sup> ATP and 15 mmol l<sup>-1</sup> phosphocreatine and leupeptin at pH 6.8. Skeletal and cardiac muscle samples were then teased apart into individual fibre bundles using sharp forceps and placed into 1 ml of fresh relaxant buffer with 0.05 mg of saponin. Fibres were gently shaken at 4°C for 30 min and were then transferred into ice-cold respiration assay medium (0.5 mmol l<sup>-1</sup> EGTA, 3 mmol l<sup>-1</sup> MgCl<sub>2</sub>, 60 mmol l<sup>-1</sup> potassium lactobionate, 700 mmol l<sup>-1</sup> sucrose, 20 mmol l<sup>-1</sup> taurine, 10 mmol l<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub> and 1 mg ml<sup>-1</sup>

BSA in 20 mmol l<sup>-1</sup> Hepes, pH 7.1) and mixed gently at 4°C for 5 min ( $\times 3$ ) to wash out saponin and ATP. Muscle fibre preparations were then blotted dry on Kimwipes (Kimtech) and weighed for use in mitochondrial respiration assays.

### Mitochondrial respiration and ROS (H<sub>2</sub>O<sub>2</sub>) production

Mitochondrial respiration was measured in control (heart,  $N=5$ ; skeletal muscle,  $N=5$ ) and 4 month aestivating (heart,  $N=7$ ; skeletal muscle,  $N=6$ ) animals. Respiration rates of cardiac and skeletal muscle mitochondria were measured using two OROBOROS O2K Oxygraphs (Anton Paar, Graz, Austria) with custom-made fluorimeters as previously described (Hickey et al., 2012). This method allows H<sub>2</sub>O<sub>2</sub> signal amplification and integration with both oxygen concentration and flux signals in DATLAB 4.3 software. All respiratory measurements of permeabilised muscle fibres were conducted at 23°C in a 2 ml chamber containing respiration assay medium at air saturation. ROS production was determined by measuring H<sub>2</sub>O<sub>2</sub> production using a horseradish peroxidase-linked Amplex Ultra Red fluorometric assay (Life Technologies, Mulgrave, VIC, Australia). Superoxide dismutase (10 U), horseradish peroxidase (10 U) and Amplex Ultra Red (12.5  $\mu$ mol l<sup>-1</sup> final concentration) were added to each chamber. To calibrate the fluorometer, 0.94 nmol of H<sub>2</sub>O<sub>2</sub> was added to each chamber before each assay. Substrates were titrated into each respiration chamber using an integrated controlled injection pump (TIP Oroboros Instruments, Schöpfstrasse, Innsbruck, Austria). A substrate cocktail was used to mimic the flow of substrates *in vivo*. However, we note that there are no data regarding mitochondrial substrate levels for *C. alboguttata* and we cannot rule out the possibility that a particular substrate(s) is used preferentially as an energy source in aestivating skeletal muscle. Whereas respiratory quotient data suggest fatty acids are the preferred substrate for aestivating frogs (van Beurden, 1980), other studies on aestivating animals show that energy may be derived from other sources (carbohydrates, ketone bodies) (Frick et al., 2008a; Frick et al., 2008b). The cocktail consisted of complex I-NADH linked substrates (pyruvate 400  $\mu$ mol l<sup>-1</sup> and malate 200  $\mu$ mol l<sup>-1</sup>), which were added in conjunction with the complex II substrate succinate (400  $\mu$ mol l<sup>-1</sup>). Substrates were titrated in stepwise additions of 5 $\times$ 0.5  $\mu$ l, 5 $\times$ 1  $\mu$ l and 5 $\times$ 3  $\mu$ l injections with a 2 min delay between injections (15 injections in total). This initiated non-phosphorylating, 'resting' mitochondrial respiration (LEAK<sub>N</sub>). Initial concentrations were 0.05 mmol l<sup>-1</sup> (malate) and 0.1 mmol l<sup>-1</sup> (pyruvate and succinate), while the final concentrations were 2.25 mmol l<sup>-1</sup> (malate) and 4.5 mmol l<sup>-1</sup> (pyruvate and succinate). Following the titration protocol with malate, pyruvate and succinate, excess ADP was added to the chamber to initiate oxidative phosphorylation (OXPHOS). Finally, the complex III inhibitor antimycin A was added to the chamber to inhibit mitochondrial respiration and determine background respiratory flux. Rates of steady-state H<sub>2</sub>O<sub>2</sub> production were traced using DATLAB 4.3. The average background rate of H<sub>2</sub>O<sub>2</sub> across experiments before the introduction of tissue to the chamber was 0.04 nmol s<sup>-1</sup> ( $\pm 0.01$  s.e.m.). Rates were corrected for tissue mass and background activity prior to analysis. We also divided the amount of H<sub>2</sub>O<sub>2</sub> formed by O<sub>2</sub> to provide an indication of the %ROS of O<sub>2</sub> (i.e. %efficiency).

### Statistics

SVL, blood glucose concentration, whole-animal metabolic rate and mitochondrial respiration during OXPHOS were analysed by one-way analysis of variance (ANOVA). The mass of gastrocnemius muscle was analysed using analysis of covariance (ANCOVA), with SVL as the covariate. Mitochondrial respiration was fitted with a Michaelis–Menten model in GraphPad Prism. Maximal respiratory flux ( $V_{max}$ ) and  $K_m$  (the substrate concentration at which respiratory flux was half  $V_{max}$ ) values were compared between 4 month aestivating and control frogs using an extra sum-of-squares *F*-test. Because H<sub>2</sub>O<sub>2</sub> production did not closely follow a Michaelis–Menten model during LEAK<sub>N</sub>, H<sub>2</sub>O<sub>2</sub> production was tested for significance using individual *t*-tests at each separate mitochondrial substrate injection point (data sets were assessed for normality and constancy of variance). H<sub>2</sub>O<sub>2</sub> production data during OXPHOS were non-normally distributed and analysed using a Wilcoxon rank sum test. The %H<sub>2</sub>O<sub>2</sub> of O<sub>2</sub> was also analysed using individual *t*-tests. All statistical tests were performed with the statistical programs R (www.r-project.org) and/or GraphPad Prism, with  $P \leq 0.05$  deemed statistically significant. Data are presented as means  $\pm$  s.e.m.



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## Competing interests

The authors declare no competing financial interests.

## Author contributions

B.D.R. and A.J.R.H. conceived and designed the study and collected and analysed the data; B.D.R. wrote the paper; A.J.R.H., C.E.F. and R.L.C. helped in editing and revising the manuscript. B.D.R., A.J.R.H. and C.E.F. approved the final version of the manuscript.

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