The Journal of Experimental Biology 214, 3782-3789

doi:10.1242/jeb.054148

RESEARCH ARTICLE

Influence of elevated temperature on metabolism during aestivation: implications for muscle disuse atrophy

Karen M. Young, Rebecca L. Cramp, Craig R. White and Craig E. Franklin*

School of Biological Sciences, The University of Queensland, Brisbane, Queensland 4072, Australia

*Author for correspondence (c.franklin@uq.edu.au)

Accepted 30 August 2011

SUMMARY

Reactive oxygen species (ROS), produced commensurate with aerobic metabolic rate, contribute to muscle disuse atrophy (MDA) in immobilised animals by damaging myoskeletal protein and lipids. Aestivating frogs appear to avoid MDA in part by substantially suppressing metabolic rate. However, as ectotherms, metabolic rate is sensitive to environmental temperature, and the high ambient temperatures that may be experienced by frogs during aestivation could in fact promote MDA. In this study, we investigated the effect of temperature on the metabolic rate of the aestivating frog *Cyclorana alboguttata* and its skeletal muscles in order to determine their likely susceptibility to MDA. Compared with non-aestivating frogs, a significant decrease in metabolic rate was recorded for aestivating frogs at 20, 24 and 30°C. At 30°C, however, the metabolic rate of aestivating frogs was significantly higher, approximately double that of frogs aestivating at 20 or 24°C, and the magnitude of the metabolic depression was significantly reduced at 30°C compared with that at 20°C. Temperature effects were also observed at the tissue level. At 24 and 30°C the metabolic rate of all muscles from aestivating frogs was significantly depressed compared with that of muscles from non-aestivating frogs. However, during aestivation at 30°C the metabolic rates of gastrocnemius, sartorius and cruralis were significantly elevated compared with those from frogs aestivating at 24°C. Our data show that the metabolism of *C. alboguttata* and its skeletal muscles is elevated at higher temperatures during aestivation and that the capacity of the whole animal to actively depress metabolism is impaired at 30°C.

Key words: aestivation, amphibian, muscle disuse atrophy, dormancy, oxygen consumption, skeletal muscle

INTRODUCTION

Temperature is a pervasive and heterogeneous environmental factor that impacts upon organisms at all levels of biological organisation. For ectotherms, environmental temperature plays a large role in determining their body temperature and the rate of their physiological processes. Most temperature-sensitive rates or processes operate within a relatively narrow thermal range whereby the performance of these traits declines outside of this thermal window (Bullock, 1955; Huey and Kingsolver, 1993; Angilletta, 2009). However, physiological traits can often be modified in response to changes in temperature, allowing an animal to respond to or compensate for changes in their thermal environment, e.g. acclimatisation (Wilson and Franklin, 1999; Angilletta, 2009).

Arid and semi-arid environments are typified by highly seasonal and often unpredictable rainfall (Schwinning and Osvaldo, 2004), temperature extremes and marked thermal variability. In these environments, many species of amphibian have evolved a suite of behavioural, physiological and biochemical adaptations, broadly termed aestivation, which allow them to avoid desiccation and survive in an environment that places considerable abiotic pressure on physiological systems (Pinder et al., 1992). Most aestivating frogs moderate their exposure to the harsh external environment by burrowing underground (Bentley, 1966; Pinder et al., 1992); however, even at depths of between 10 and 40 cm below the surface, where burrowing frogs can be found (Thompson et al., 2005; Cartledge et al., 2006), temperatures can still exceed 30°C for extended periods (see Fig. 1) (Shoemaker et al., 1972; Claussen, 1974; McLanahan et al., 1976; Loveridge and Craye, 1979).

Metabolic depression is a conserved feature of all dormancy strategies, including aestivation, and is coordinated across whole-animal, tissue and molecular levels in order to protect endogenous fuel supplies from premature exhaustion (Cowan and Storey, 1999; Cowan et al., 2000; Storey, 2002; Storey and Storey, 2004). In addition, metabolic depression is thought to protect the muscles of (immobile) dormant animals from muscle disuse atrophy (MDA) in part *via* a decrease in the production of reactive oxygen species (ROS) (Hudson and Franklin, 2002) because ROS are produced in proportion to metabolic rate (Adelman et al., 1988; Turrens, 2003). MDA results in morphological changes that may include reduced cross-sectional area of muscles and/or muscle fibres, fibre-type switching and increased connective tissue and which may result in reduced muscle function and performance (Appell, 1990; Boonyarom and Inui, 2006; Clark, 2009). MDA has been linked to an accumulation of ROS, which causes oxidative stress and damage to muscle tissue (Kondo et al., 1991; Powers et al., 2007). Indeed, a comparison of dormant animals has shown that those with lower mass-specific metabolic rates suffer less MDA over a normalised period of disuse (Hudson and Franklin, 2002). Although the muscles of aestivating frogs are remarkably tolerant of MDA compared with those of other animals (Hudson and Franklin, 2002), previous studies have shown that in *Cyclorana alboguttata* different skeletal muscles undergo different degrees of atrophy, displaying different patterns of morphological change (Mantle et al., 2009) and differentially

regulated metabolic enzymes (Mantle et al., 2010). This suggests that, given the links between metabolic rate and ROS production, the rate of oxygen consumption and metabolic depression of muscles during aestivation may also be muscle specific. As frogs are ectotherms, temperature-related changes in metabolic rate have the potential to increase rates of MDA in dormant immobilised frogs, presumably in a muscle-specific fashion, and impact upon post-emergence locomotor performance and survival. In fact, the metabolic rate of the toad *Scaphiopus couchii* appears to be more sensitive to temperature during aestivation than while active (Seymour, 1973), suggesting that temperature-induced rates of ROS production may even be higher in the tissues of aestivating animals relative to non-aestivating animals.

In the present study we sought to determine the effect of environmental temperature on the metabolic rate and the magnitude of the metabolic depression of aestivating green-striped burrowing frogs, *C. alboguttata*, at both the whole-animal and tissue (muscle) levels. We hypothesised that high temperatures during aestivation would increase the metabolic rate of frogs and tissues, and the extent of metabolic depression associated with aestivation would be compromised at higher temperatures. Moreover, we predicted that the metabolic responses of muscles to aestivation would be muscle specific in a way that complements the differential susceptibility of specific muscles to MDA in aestivating *C. alboguttata* found in previous studies. Following on from this we further hypothesised that the metabolic responses of muscles to temperature during aestivation would also be muscle specific.

MATERIALS AND METHODS

Experimental animals

Animals were collected in accordance with State and Federal laws pertaining to the use of animals for scientific purposes (Scientific Purposes Permit number WISP03572406). All experiments were carried out with the approval of the University of Queensland's Animal Welfare Committee (permit numbers SIB/442/07/UQ and SIB/602/08/ARC).

Following heavy rains, green striped burrowing frogs, *C. alboguttata* Günther, were collected from agricultural areas in south-east Queensland, Australia. Frogs were transported to The University of Queensland and maintained in individual housing, which was cleaned weekly. Frogs were fed once a week with live food (crickets or cockroaches). Frogs were assigned randomly to treatment temperatures of 20, 24 or 30°C for whole-animal metabolic rate (oxygen consumption rate, $\dot{V}_{O2}$) determination, and to 24 or 30°C for muscle $\dot{V}_{O_2}$ determination. Treatment temperatures were within the range of soil temperatures frogs would be expected to experience in the wild based on data from temperature loggers buried at 10, 20 and 40 cm depth in areas with known *C. alboguttata* populations (Fig. 1).

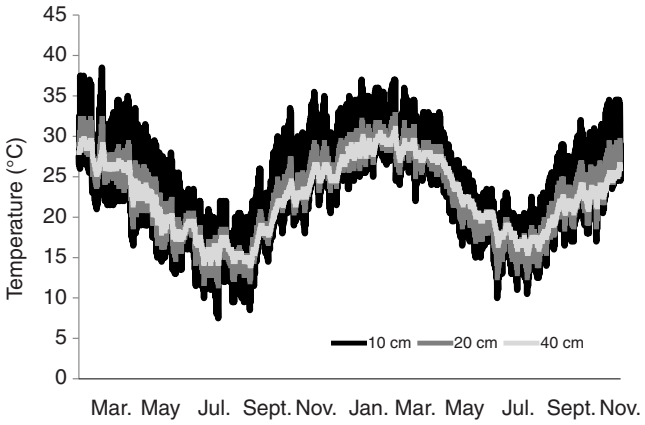

Fig. 1. Temperature profiles (2008–2009) from data loggers positioned in soil/clay at depths of 10, 20 and 40 cm, in a sun-exposed site in Theodore (Queensland, Australia) where *Cyclorana alboguttata* were collected.

Determination of whole-animal $\dot{V}_{O_2}$

Frogs were maintained in controlled-temperature rooms at their treatment temperatures for 2 weeks without food prior to beginning the measurements. Mass-specific $\dot{V}_{O_2}$ was measured using closed-system respirometry (N=10 per treatment group). Frogs were placed into individual respirometers (550–1020 ml volume) with moist paper towels 24 h prior to the first measurements and left in darkened conditions. Aestivation was induced by allowing the paper towelling to slowly dry out (see Flanigan et al., 1991; Withers, 1993; Fuery et al., 1998; Withers and Thompson, 2000; Bayomy et al., 2002; Kayes et al., 2009a). To prevent dehydration, small amounts of water (~0.5 ml) were added weekly to the paper in each respirometer. Animal behaviour (adoption of water-conserving posture and formation of cocoon) and condition were monitored under a red light. Any frogs showing signs of stress or those not entering aestivation were removed from the experiment.

Metabolic rate measurements were made over the course of a 10 week period as animals entered aestivation and these measurements occurred in weeks 1, 5, 8 and 10. Week 1 measurements consisted of four trials (repeated measures) to obtain a mean 'resting' metabolic rate for each frog. In order to minimise disturbance during aestivation only one measurement of metabolic rate per animal was made in weeks 5, 8 and 10. Measurements were made by sealing respirometers and withdrawing a 25 ml sample of air through a 3-way stopcock in the lid of the respirometer. Air samples were injected through a Drierite column and into an ML205 O_2/CO_2 gas analyser (ADInstruments, Castle Hill, NSW, Australia) connected to a Powerlab (ADInstruments) and computer. The fractional concentrations of O_2 and CO_2 in the gas sample were recorded using Chart software v5 (ADInstruments). Respirometers remained sealed for between 4 and 23 h dependent on temperature and time in aestivation (4–5 h for resting, non-aestivating frogs, 14–23 h for aestivating frogs), before a final 25 ml sample of air was withdrawn. The rate of O_2 consumption by the animal (in $\mu l\ O_2\ g^{-1}\ h^{-1}$) was then calculated according to Vleck (Vleck, 1987):

$$\mathrm{RER} = \frac{F_{\mathrm{end\ CO_2}} - F_{\mathrm{start\ CO_2}}}{F_{\mathrm{start\ O_2}} - F_{\mathrm{end\ O_2}}},$$

$$\dot{V}_{\mathrm{O_2}} = \frac{V \times \left(F_{\mathrm{start\ O_2}} - F_{\mathrm{end\ O_2}}\right)}{1 - \left(1 - \mathrm{RER}\right) \times F_{\mathrm{start\ O_2}}} \div t, \qquad (1)$$

$$\dot{V}_{\mathrm{CO_2}} = \mathrm{RER} \times \dot{V}_{\mathrm{O_2}},$$

where RER is the respiratory gas exchange ratio and F_{start} and F_{end} are the fractional content of the gases CO_2 or O_2 in the start and end gas samples. V denotes the volume of the respirometry chamber minus the volume of added water and animal mass (assuming a density of $1\ ml\ g^{-1}$) and t is the time the chamber was sealed. Metabolic rates were standardised to a 20 g animal using an allometric scaling exponent of 0.73 (see Kayes et al., 2009a). Background oxygen consumption was corrected for using values obtained from blank respirometers (i.e. containing no frog).

Determination of skeletal muscle oxygen consumption rates

The metabolic rate of isolated skeletal muscles was measured in 24°C non-aestivating (N=7), 24°C 6 month aestivating (N=7), 30°C non-aestivating (N=7) and 30°C 6 month aestivating (N=9) frogs. In order to facilitate 6 months of aestivation, frogs were placed in 41 ice cream containers filled with wet clay mud. Frogs were then left in the dark in constant temperature rooms at their respective treatment temperature. Frogs were checked once a week and were deemed to be in aestivation once they had burrowed into the mud block and remained there. The mud was allowed to dry slowly over the course of the experimental period. Note that, unlike for animals aestivating in respirometers, no data were collected at 20°C as animals would not remain burrowed or enter aestivation. Non-aestivating animals were maintained at their treatment temperature and were fed and cleaned weekly. After 6 months, aestivating frogs (removed from their clay burrows) and non-aestivating frogs from each temperature group were immediately killed by double pithing. Five skeletal muscles were dissected from the left hindleg of each frog: gastrocnemius, sartorius, iliofibularis, gluteus magnus and cruralis. Each muscle was weighed immediately and placed in air-saturated Ringer solution (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, 5 mmol l^{-1} Hepes, 10 mmol l^{-1} glucose, adjusted to pH 7.4 with NaOH). All solutions were maintained, and experiments run, at the treatment temperature of the frog that the tissues were obtained from. Each muscle was cut longitudinally into thin slices (<0.5 mm) with a razor blade.

Rates of tissue oxygen consumption were measured using a 24-channel oxygen meter (sensor dish® reader, SDR2, PreSens GmbH, Regensburg, Germany). Vials (5 ml) were equipped with a planar oxygen-sensing spot (5 mm diameter, type Sp-PSt5-NAU-D5-YOP, PreSens GmbH) that measured oxygen (% air saturation) *via* optical fluorescence. The vials were randomly distributed in a 24-well rack secured to the SDR2. The SDR2 was placed on an orbital shaker set at 288 r.p.m. in a temperature-controlled chamber (set to the treatment temperature of the source frog) and connected to a computer. Recordings were made every 2 min for vials of oxygenated Ringer solution, in the absence of tissue, to allow the oxygen concentration reading to stabilise. Tissue slices were then added to the vials, suspended above the sensor in a small soft mesh basket, and vials were then topped up with oxygenated Ringer solution and sealed. Vials containing Ringer solution only served as blanks to correct for background oxygen consumption. Oxygen concentration readings were recorded every 2 min until muscle respiration rate stabilised. All measures of tissue oxygen consumption were made at the treatment temperature of the frog from which the tissues were collected. At the termination of the experiment tissue slices were removed from vials, gently blotted on paper towels and weighed. All tissues were handled in a standardised manner throughout all experimental recordings.

Calculation of tissue $\dot{V}_{O_2}$

Tissue slice $\dot{V}_{O_2}$ values were calculated using the maximum slopes obtained from time-dependent oxygen consumption rates recorded in tissue vials and blank vials using the following equation:

$$\dot{V}_{O_2} = -1 \times [(m_t - m_b) / 100] \times V \times \beta_{O_2}, \quad (2)$$

where m_t is the maximum slope recorded for individual tissues, m_b is the mean slope recorded for all blanks, V is the volume of the vial (5 ml), and β_{O_2} is the O_2 capacitance of fully oxygenated Ringer solution at the appropriate experimental temperature (6.025 ml l^{-1} at 24°C, 5.391 ml l^{-1} at 30°C). Oxygen capacitance values were initially taken for oxygen solubility in water (mg l^{-1}) at the appropriate temperature [from Weiss, cited in Lewis (Lewis, 2006)] then corrected for the conductivity of the Ringer solution (13.8–14 mS cm^{-1}) using correction factors of 0.954 and 0.956, for 24 and 30°C, respectively (Lewis, 2006), prior to conversion to ml l^{-1}. Data recorded below 50% air saturation were not used. All tissue metabolic rates are expressed as µl O_2 g^{-1} h^{-1}.

Statistical analysis

Data are presented as means ± s.e.m. Whole-animal ($\dot{V}_{O_2}$) data were tested between temperature groups using one-way ANOVA for each time point. Data within temperature treatment groups were compared between consecutive time points using matched pair analysis. Magnitude (%) of metabolic depression data were arcsine transformed and the difference between temperature groups tested using one-way ANOVA. Tissue $\dot{V}_{O_2}$ data were log transformed to meet equality of variance assumptions. A two-way ANOVA model was fitted to the transformed data considering temperature (two levels: 24 and 30°C), metabolic state (two levels: controls and aestivators), and the interaction between temperature and metabolic state as main effects. Frog snout–vent length (SVL) was included as a covariate. The model was run for each muscle separately. Where significant effects were found they were localised with Tukey's honestly significantly different (HSD) test. Planned comparisons between 24 and 30°C within the aestivating group were run for each muscle using Student's t-test or Welch ANOVA where appropriate. Muscle-specific $\dot{V}_{O_2}$ within treatments was tested using a general linear model considering muscle type as the main effect with source animal as a random effect to account for the non-independence of data when analysing data for different muscles obtained from the same animal. Where general linear model analyses detected significant differences, they were localised using Tukey HSD. All analyses were conducted using JMPIN v4 or JMP v8 software with significance set at P<0.05.

RESULTS

The body mass of frogs used in this study ranged from 19.6 to 41.1 g (mean 27.2±0.9 g) and SVL of these animals ranged from 51.05 to 63.04 mm (mean 56.8±0.6 mm).

Whole-animal $\dot{V}_{O_2}$

In all temperature treatments (20, 24 and 30°C), *C. alboguttata* exhibited a significant decrease in $\dot{V}_{O_2}$ over the 10 week measurement period, which corresponded to a cessation of movement and the formation of a cocoon, typical of aestivation in this species (20°C P=0.0024, 24°C P=0.001, 30°C P=0.002; Fig. 2). The highest metabolic rates were recorded from non-aestivating, resting frogs at week 1. By week 5, the $\dot{V}_{O_2}$ of *C. alboguttata* had decreased significantly, and it continued to decrease in the following weeks, with the lowest mean metabolic rate observed after 10 weeks in all groups (Fig. 2).

The metabolic rate of *C. alboguttata* was significantly influenced by temperature at rest and during aestivation across the three temperature treatments (ANOVA, P<0.001; Fig. 2 and Fig. 3A). Interestingly, however, temperature did not have a significant effect on the $\dot{V}_{O_2}$ of frogs between 20 and 24°C during the 10 week sampling period (Tukey HSD, P>0.05; Fig. 2). At rest (week 1, Fig. 2), the $\dot{V}_{O_2}$ of *C. alboguttata* at 30°C was significantly higher than the $\dot{V}_{O_2}$ of frogs at 20 and 24°C and these frogs maintained the highest oxygen consumption rates during aestivation, relative to animals at the lower temperatures (weeks 5, 8 and 10; Fig. 2 and Fig. 3A). The mean minimum $\dot{V}_{O_2}$ of aestivating frogs recorded at 30°C was 22.3±1.4 µl O_2 g^{-1} h^{-1}, which was more than double that of aestivating frogs at 20°C ($\dot{V}_{O_2}$=9.9±1.6 µl O_2 g^{-1} h^{-1}; P<0.01; Fig. 3A).

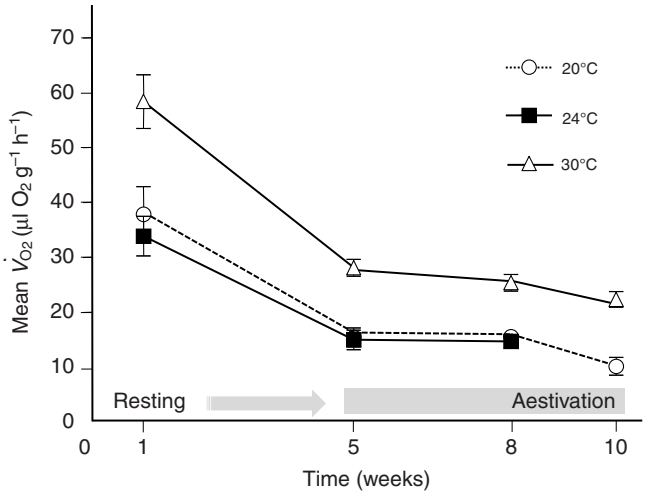

Fig. 2. The effect of temperature on the mean rate of whole-animal oxygen consumption ($\dot{V}_{O_2}$, µl O_2 g^{-1} h^{-1}, normalised to a body mass of 20 g) of *C. alboguttata* at rest (week 1) and whilst in aestivation (weeks 5, 8 and 10). Results are means ± s.e.m. (*N*=9–10).

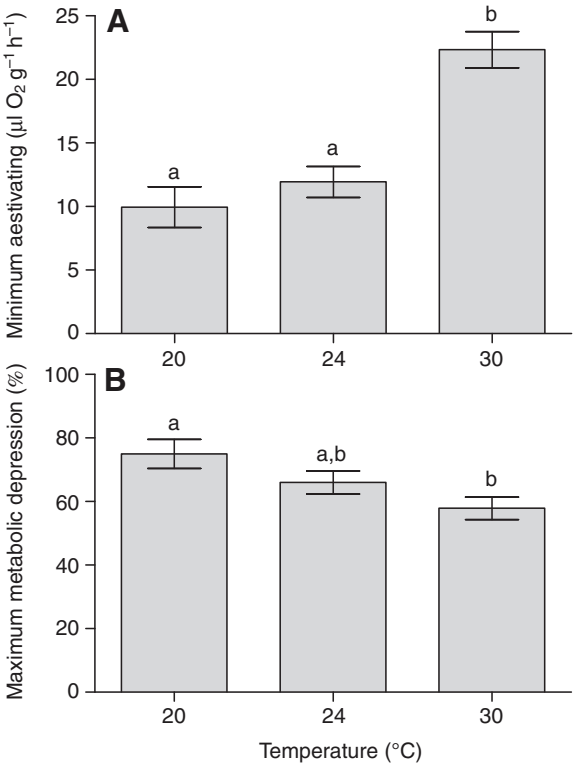

Fig. 3. The effect of temperature on (A) the mean minimum rate of oxygen consumption ($\dot{V}_{O_2}$) of aestivating *C. alboguttata*, and (B) the mean maximum metabolic depression exhibited by frogs in aestivation. Results are means ± s.e.m. (*N*=9–10). Different letters indicate significant difference at *P*<0.05.

Exposure to a higher temperature decreased the magnitude of the maximum metabolic depression that was achievable during aestivation by *C. alboguttata* (Fig. 3B). Despite the frogs at 30°C being inactive and encased in a cocoon, they were only able to depress their metabolic rate by 58±3% relative to resting levels. In contrast, at 20 and 24°C, the frogs were able to depress their metabolic rate by 75±5% and 66±4%, respectively.

Skeletal muscle

The covariate SVL was not significant in any model. The $\dot{V}_{O_2}$ of tissue slices recorded from five hindlimb muscles from aestivating animals was always significantly lower than that recorded from non-aestivating animals, indicating that tissue-level metabolic depression is associated with aestivation in *C. alboguttata* (Fig. 4). Temperature had a significant effect on all muscles except the iliofibularis, with rates of oxygen consumption significantly higher at 30°C than at 24°C (Fig. 4). There was a significant interaction between temperature and metabolic state for the gastrocnemius ($F_{1,24}$=15.0627, *P*=0.0007) and cruralis ($F_{1,24}$=6.6197, *P*=0.0167) muscles whereby the rates of oxygen consumption in muscles from frogs aestivating at 30°C were substantially higher than those of frogs aestivating at 24°C (all *P*<0.05, Fig. 4).

Planned comparisons between the $\dot{V}_{O_2}$ of muscles from frogs aestivating at 24 and 30°C demonstrated that oxygen consumption was significantly higher in the cruralis ($F_{1,13}$=17.7526, *P*=0.001), gastrocnemius (Welch $F_{1,8.6981}$=33.7527, *P*=0.0003), and sartorius ($F_{1,13}$=5.0472, *P*=0.0427) muscles from frogs aestivating at 30°C relative to those from frogs aestivating at 24°C. The $\dot{V}_{O_2}$ of the gluteus magnus was also consistent with this pattern but just failed to reach significance (Welch $F_{1,11.249}$=4.7816, *P*=0.0507). The rate of oxygen consumption of the iliofibularis from aestivating frogs was not influenced by temperature at all (Welch $F_{1,8.1251}$=1.0821, *P*=0.3282).

Differences in the rates of oxygen consumption were also apparent between muscle types in non-aestivating frogs at both 24°C ($F_{4,23}$=9.0809, *P*=0.0001) and 30°C ($F_{4,22}$=5.5158, *P*=0.0031) and in frogs aestivating at 24°C ($F_{4,20}$=24.0352, *P*<0.0001). There was, however, no difference between the rates of oxygen consumption of the five muscles from frogs aestivating at 30°C ($F_{4,30}$=0.7299, *P*=0.5787). The rates of oxygen consumption in the smaller muscles (iliofibularis and sartorius) were significantly higher than those of the larger muscles (cruralis and gastrocnemius) (Fig. 4). The gluteus magnus showed a rate of oxygen consumption that was intermediate between the small and large muscles.

DISCUSSION

For *C. alboguttata* the impacts of environmental temperature fluctuations are somewhat alleviated by burrowing underground and selecting a thermally buffered microhabitat. Although we have relatively little information regarding natural burrow depths of wild *C. alboguttata*, studies of closely related cocoon-forming frog species (*Cyclorana australis* and *Cyclorana platycephala*) have recorded natural burrow depths of between 2 and 30 cm (Van Beurden, 1984; Tracy et al., 2007). From our laboratory studies, this burrow depth range seems likely to also reflect burrowing tendencies of *C. alboguttata*. From Fig. 1 it is clear that at shallower depths *C. alboguttata* would be subject to large daily fluctuations in temperature. At depth, extreme fluctuations are dampened and temperature is more constant; however, even at 40 cm below the surface, soil temperatures can reach 30°C or more and can remain elevated for some time. The thermal regime employed in this study is therefore realistic and may even be somewhat conservative depending on frog burrow depths. Although some non-cocoon-forming species can continue to burrow over the course of their underground aestivation, presumably to follow the soil moisture level, this would seem

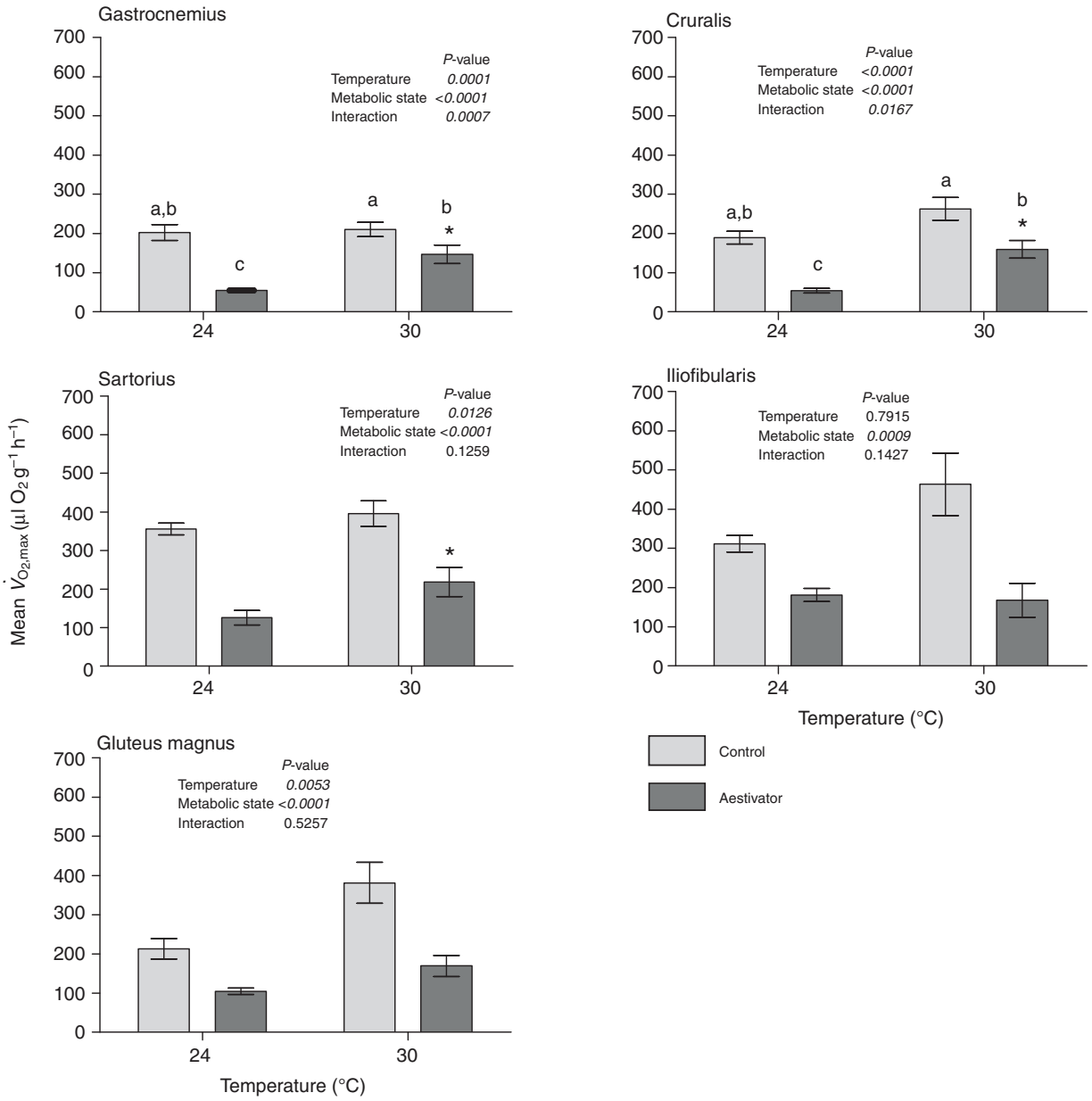

Fig. 4. Maximal rate of oxygen consumption ($\dot{V}_{O_2}$) of skeletal muscles from *C. alboguttata*. Data are expressed as means ± s.e.m. *P*-values are given in each graph (significant values are in italics). *Significant difference in oxygen consumption of muscle between aestivating (dark grey bars) treatments. Letters indicate differences between columns where the interaction of temperature and metabolic rate was significant. Sample sizes within each graph, from left to right, are: gastrocnemius *N*=7, 6, 7, 9; cruralis *N*=7, 6, 7, 9; sartorius *N*=7, 7, 6, 8; iliofibularis *N*=6, 7, 6, 8; gluteus magnus *N*=7, 5, 7, 9.

unlikely to occur in cocoon-forming species like *C. alboguttata* burrowed in heavy clay soils, which become impenetrable as the soils dry and frogs would remain subjected to the temperature profile of the depth at which they had burrowed.

During aestivation many species of frog substantially reduce their metabolic rate by between 60% and 80% (Seymour, 1973; Flanigan et al., 1991; Withers, 1993) and the magnitude of metabolic depression observed for aestivating *C. alboguttata* in the present study was consistent with this. These results are also consistent with earlier studies which have shown that as ambient temperature increases, the rate of oxygen consumption by aestivating frogs also increases (Van Beurden, 1980), and the magnitude of metabolic depression achieved decreases (Seymour, 1973; Abe and Garcia, 1991). Although aestivating *C. alboguttata* significantly depressed metabolic rate at all experimental temperatures, their capacity to do so was clearly impaired at 30°C. As expected, exposure to higher temperatures during aestivation resulted in higher rates of oxygen consumption, which reduced the ability of frogs aestivating at 30°C to suppress metabolic rate to the same extent as animals aestivating at 20°C. Thus, temperature may have a more profound influence on physiological function during aestivation as opposed to while active (not aestivating), consistent with the results of Seymour (Seymour, 1973). The inability of frogs to suppress metabolic rate at 30°C to the same rate of oxygen consumption shown by an animal at 20°C would mean that the energy costs of maintaining physiological function are greater in frogs aestivating at the higher

temperature, and during long periods of aestivation these costs could significantly impact their ability to survive.

Interestingly, some species of aestivating frogs, such as *Leptodactylus fuscus* and *Physalaemus fuscomaculatus*, show whole-animal oxygen consumption rates that are thermally insensitive between 20 and 25°C (Abe and Garcia, 1991). Thus, the lack of difference in the metabolic rate of non-aestivating and aestivating *C. alboguttata* kept at 20 and 24°C may also reflect some similar thermal insensitivity of oxygen consumption rate within this temperature range. This may allow for subtle fluctuations in environmental temperature about this range to be 'absorbed' to provide some physiological stability. However, at 30°C the effect of environmental temperature on the physiological rate processes of aestivating *C. alboguttata* was apparently unavoidable and consequently oxygen consumption rates were substantially elevated and remained so, which is probably reflective of the physiological costs of maintaining essential functions at higher temperature.

Although during aestivation at 24°C *C. alboguttata* skeletal muscles undergo a slow yet selective atrophy (Hudson et al., 2006; Symonds et al., 2007; Mantle et al., 2009), relative to other animals aestivating frogs suffer little MDA and it is postulated that this is related to the protection from ROS damage afforded by metabolic depression (Hudson and Franklin, 2002). Metabolic depression during aestivation is a coordinated strategy that is reflected across levels of biological organisation from whole-animal to tissue and sub-cellular levels (Storey, 1988; Storey and Storey, 1990; Cowan and Storey, 1999; Cowan et al., 2000; Kayes et al., 2009b). In the present study, all isolated muscles examined from aestivating *C. alboguttata* demonstrated substantially suppressed rates of oxygen consumption. Rates of oxygen consumption by the gastrocnemius, cruralis and sartorius muscles were, however, significantly higher in frogs aestivating at 30°C than in frogs aestivating at 24°C, suggesting that these muscles may be more susceptible to MDA as a consequence of increased ROS production (Hudson and Franklin, 2002) proportional to metabolic rate (Adelman et al., 1988; Turrens, 2003). However, aestivation temperature clearly did not influence the metabolic rates of all muscles in the same way, as the metabolic rate of the iliofibularis and, to a lesser extent, the gluteus magnus, was unaffected by aestivation temperature. This implies that the muscles examined are differentially affected by temperature and/or aestivation and this may reflect muscle-specific differences in their underlying physiochemical properties, which in turn might influence a muscles' predisposition for atrophy during aestivation.

Muscle-specific differences in their metabolic responses to temperature probably reflect underlying morphological and/or physiological differences between muscles and/or muscle-specific alterations to metabolic pathways that accompany aestivation. In the present study, the iliofibularis and sartorius muscles generally consumed significantly more oxygen (per gram of tissue) than the gastrocnemius, gluteus magnus and cruralis, and previous studies have shown that differences in the metabolic physiology of isolated skeletal muscles may reflect their functional similarity (Duellman and Trueb, 1994; Lutz et al., 1998; Mantle et al., 2009). Indeed, Mantle and colleagues found that changes in key metabolic enzymes (cytochrome *c* oxidase, citrate synthase and lactate dehydrogenase) were consistent within the functional classification of 'non-jumping' (iliofibularis and sartorius) and 'jumping' (gastrocnemius and cruralis) muscles, but were distinctly different from each other (i.e. between jumping and non-jumping), suggesting that muscles which perform similar functions display similar metabolic profiles and are similarly affected by aestivation (Mantle et al., 2010). However, muscle fibre-type composition is unlikely to be related to differences in the metabolic rate of muscles and their differential responses to temperature as recent work has suggested that fibre composition does not necessarily predict muscle metabolic capacity (Crockett and Peters, 2008). Similarly, anuran muscles can also show vastly different metabolic and contractile properties despite having similar fibre compositions (Putnam and Bennett, 1983).

In addition to underlying muscle-specific differences, aestivation itself may promote differential changes in muscles that alter their metabolic profiles and responses to temperature. Cellular metabolic enzyme and signal transduction pathway changes associated with dormancy have been shown to occur in a tissue-/organ-specific fashion in aestivating spadefoot toads, whereby hindlimb skeletal muscle undergoes a much more profound reorganisation than does either the brain or liver (Cowan et al., 2000). Moreover, previous studies have shown that aestivation alters the activity of various metabolic enzymes in the skeletal muscles of *C. alboguttata*, and that different muscles show different degrees of biochemical change (Mantle et al., 2010). Changes in the metabolic physiology of skeletal muscles may involve modification of glycolytic (reversible phosphorylation of regulatory enzymes) and/or oxidative pathways (mitochondrial substrate metabolism and oxidative phosphorylation) (for a review, see Storey, 1997) [for aestivating anurans, see studies by Cowan and colleagues (Cowan and Storey, 1999; Cowan et al., 2000)]. In addition, enzymes involved in the regulation of metabolic suppression in hibernating mammals show different kinetic responses to temperature in dormant and awake animals (see Storey, 1997), suggesting changes at the biochemical level occur in some proteins during dormancy that may affect their thermal responsiveness and alter the metabolic properties of tissues.

Higher rates of tissue oxygen consumption during aestivation at high temperatures have the potential to enhance rates of MDA in *C. alboguttata* as a result of the relationship between metabolic rate and ROS production, and ROS production and MDA (Powers et al., 2005; Powers et al., 2007). Although the hypometabolic state during aestivation probably affords some protection against MDA by lowering the production of ROS (Hudson and Franklin, 2002), previous studies have shown that tissues of aestivating anurans still do accumulate ROS damage (Grundy and Storey, 1998). Aestivating land snails, *Otala lactea*, show an increase in oxidative damage to lipids when the rate of oxygen consumption increases (Hermes-Lima and Storey, 1995). The elevated oxygen consumption rates found in some skeletal muscles of aestivating *C. alboguttata* lends credence to the hypothesis that skeletal muscles may be differentially susceptible to disuse atrophy on the basis of rates of oxygen consumption. Indeed, Mantle and colleagues determined that in *C. alboguttata* the iliofibularis and sartorius muscles underwent substantially more atrophy during aestivation than either the gastrocnemius or cruralis muscles (Mantle et al., 2009), and based on the results of the present study this is consistent with the aforementioned hypothesis. Given that exposure to high temperatures accelerates ROS production in skeletal muscles of mammals (Edwards et al., 2007; Muller et al., 2007; van der Poel et al., 2007), it might be expected that the accumulation of oxidative damage during aestivation would also be exacerbated by higher aestivation temperatures. It might be predicted that the muscles from *C. alboguttata* aestivating at 30°C that had metabolic rates in excess of those observed in frogs aestivating at 24°C (i.e. the gastrocnemius, sartorius and cruralis) would be susceptible to increased oxidative damage and increased rates of MDA during aestivation at this higher temperature. Further investigations should focus on clarifying this proposition.

Given the importance of skeletal muscle immediately post-aestivation, an increase in susceptibility of some muscles to disuse atrophy at higher temperatures may have serious consequences for locomotor performance and survival, unless compensatory processes are up-regulated. Muscle protection against ROS damage may be afforded by an increase in the production of antioxidants (Gutteridge, 1995). In *C. alboguttata* aestivating at approximately 24°C, antioxidant production, normalised to metabolic rate, is up-regulated ~3.5-fold (Hudson et al., 2006). If the rate of antioxidant production is indeed related to metabolic rate in aestivating frogs, then an increase in metabolic rate at 30°C may also result in the up-regulation of compensatory antioxidants. In addition, heat-shock proteins (HSP) have a significant and highly conserved role in the protection of skeletal muscle tissue (Liu et al., 2006) and are suggested to protect tissues against MDA in rats exposed to high temperature (Naito et al., 2000). Enhanced production of antioxidants and HSP during aestivation in *C. alboguttata* would probably pose an additional energy cost and, consequently, the higher metabolic rate of muscles from frogs aestivating at 30°C may in part be due to increased energy costs associated with enhanced mobilisation of the underlying mechanisms that protect against MDA. An ability to protect muscle function despite a high ambient temperature may mean that effects on locomotor performance following aestivation are minimised.

Concluding remarks

Metabolic depression across all levels of biological organisation is a vital strategy allowing organisms to survive for extended periods in environments that become unfavourable. Higher temperatures during the aestivation period of burrowing frogs present challenges to this strategy. At both the whole-animal and tissue levels, significantly higher oxygen consumption rates during aestivation at 30°C would accelerate the use of endogenous fuel stores and would also expose frogs to a higher rate of ROS insult relative to frogs aestivating at lower ambient temperatures. At the tissue level, increases in the rate of oxygen consumption by muscles from aestivating frogs at higher aestivation temperatures and the associated increased ROS production have implications for muscle integrity. The muscle-specific differences in metabolic rate and temperature sensitivity indicate that future investigations should focus on both better elucidating the mechanisms resulting in muscle-specific effects of temperature and examining the fitness implications that this may have for organisms aestivating for prolonged periods at high temperatures.

ACKNOWLEDGEMENTS

The authors wish to thank four anonymous reviewers for their helpful comments on an earlier version of this manuscript which resulted in its improvement.

FUNDING

This work was supported by the Australian Research Council [grant DP0666256 to C.E.F.; grant DP0987626 to C.R.W. and P. B. Frappell]. K.M.Y. was supported by a University of Queensland Postgraduate Research Scholarship.

REFERENCES

Abe, A. S. and Garcia, L. S. (1991). Response to temperature in oxygen uptake of awake and dormant frogs (Amphibia, Leptodactylidae). *Stud. Neotrop. Fauna Environ.* **26**, 135-141.

Adelman, R., Saul, R. L. and Ames, B. N. (1988). Oxidative Damage to DNA: Relation to Species Metabolic Rate and Life Span. *Proc. Natl. Acad. Sci. USA* **85**, 2706-2708.

Angilletta, M. J. (2009). Thermal adaptation. A theoretical and empirical synthesis. Oxford; New York: Oxford University Press.

Appell, H. (1990). Muscular atrophy following immobilisation. *Sports Med.* **10**, 42-58.

Bayomy, M. F., Shalan, A. G., Bradshaw, S. D., Withers, P. C., Stewart, T. and Thompson, G. (2002). Water content, body weight and acid mucopolysaccharides, hyaluronidase and beta-glucuronidase in response to aestivation in Australian desert frogs. *Comp. Biochem. Physiol.* **131A**, 881-892.

Bentley, P. (1966). Adaptations of amphibia to arid environments. *Science* **152**, 619-623.

Boonyarom, O. and Inui, K. (2006). Atrophy and hypertrophy of skeletal muscles: structural and functional aspects. *Acta Physiol.* **188**, 77-89.

Bullock, T. H. (1955). Compensation for temperature in the metabolism and activity of poikilotherms. *Biol. Rev. Camb. Philos. Soc.* **30**, 311-342.

Cartledge, V. A., Withers, P. C., McMaster, K. A., Thompson, G. G. and Bradshaw, S. D. (2006). Water balance of field-excavated aestivating Australian desert frogs, the cocoon-forming *Neobatrachus aquilonius* and the non-cocooning *Notaden nichollsi* (Amphibia: Myobatrachidae). *J. Exp. Biol.* **209**, 3309-3321.

Clark, B. (2009). In vivo alterations in skeletal muscle form and function after disuse atrophy. *Med. Sci. Sports Exerc.* **41**, 1869-1875.

Claussen, D. (1974). Urinary bladder water reserves in the terrestrial toad, *Bufo fowleri*; and the aquatic frog, *Rana clamitans*. *Herpetologica* **30**, 360-367.

Cowan, K. and Storey, K. (1999). Reversible phosphorylation control of skeletal muscle pyruvate kinase and phosphofructokinase during estivation in the spadefoot toad, Scaphiopus couchii. *Mol. Cell. Biochem.* **195**, 173-181.

Cowan, K., MacDonald, J., Storey, J. and Storey, K. (2000). Metabolic reorganisation and signal transduction during estivation in the spadefoot toad. *Exp. Biol. Online* **5**, 61-85.

Crockett, C. and Peters, S. (2008). Hindlimb muscle fiber types in two frogs (*Rana catesbeiana* and *Litoria caerulea*) with different locomotor behaviors: Histochemical and enzymatic comparison. *J. Morphol.* **269**, 365-374.

Duellman, W. E. and Trueb, L. (1994). Musculoskeletal system. In *Biology of Amphibians* (ed. W. E. Duellman and L. Trueb), pp. 289-364. New York: McGraw-Hill.

Edwards, J. N., Macdonald, W. A., van der Poel, C. and Stephenson, D. G. (2007). $O2^-$ production at 37°C plays a critical role in depressing tetanic force of isolated rat and mouse skeletal muscle. *Am. J. Physiol. Cell Physiol.* **293**, C650-C660.

Flanigan, J. E., Withers, P. C. and Guppy, M. (1991). In vitro metabolic depression of tissues from the aestivating frog *Neobatrachus pelobatoides*. *J. Exp. Biol.* **161**, 273-283.

Fuery, C. J., Withers, P. C., Hobbs, A. A. and Guppy, M. (1998). The role of protein synthesis during metabolic depression in the Australian desert frog *Neobatrachus centralis*. *Comp. Biochem. Physiol.* **119A**, 469-476.

Grundy, J. E. and Storey, K. B. (1998). Antioxidant defenses and lipid peroxidation damage in estivating toads, *Scaphiopus couchii*. *J. Comp. Physiol. B* **168**, 132-142.

Gutteridge, J. M. C. (1995). Lipid-peroxidation and antioxidants as biomarkers of tissue-damage. *Clin. Chem.* **41**, 1819-1828.

Hermes-Lima, M. and Storey, K. (1995). Antioxidant defences and metabolic depression in a pulmonate land snail. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **268**, R1386-R1393.

Hudson, N. J. and Franklin, C. E. (2002). Maintaining muscle mass during extended disuse: aestivating frogs as a model species. *J. Exp. Biol.* **205**, 2297-2303.

Hudson, N. J., Lehnert, S. A., Ingham, A. B., Symonds, B., Franklin, C. E. and Harper, G. S. (2006). Lessons from an estivating frog: sparing muscle protein despite starvation and disuse. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **290**, R836-R843.

Huey, R. B. and Kingsolver, J. G. (1993). Evolution of resistance to high temperatures in ectotherms. *Am. Nat.* **142**, S21-S46.

Kayes, S. M., Cramp, R. L. and Franklin, C. E. (2009a). Metabolic depression during aestivation in *Cyclorana alboguttata*. *Comp. Biochem. Physiol.* **154A**, 557-563.

Kayes, S. M., Cramp, R. L., Hudson, N. J. and Franklin, C. E. (2009b). Surviving the drought: burrowing frogs save energy by increasing mitochondrial coupling. *J. Exp. Biol.* **212**, 2248-2253.

Kondo, H., Nakagaki, I., Sasaki, S., Hori, S. and Itokawa, Y. (1993). Mechanism of oxidative stress in skeletal muscle atrophied by immbolisation. *Am. J. Physiol. Endocrinol. Metab.* **265**, E839-E844.

Lewis, M. E. (2006). A6: Field measurements, section 6.2, Dissolved oxygen. In *National Field Manual for the Collection of Water-Quality Data* (ed. F. D. Wilde). US Geological Survey accessed from http://pubs.water.usgs.gov/twri9A6/ on 25 June 2009.

Liu, Y. F., Gampert, L., Nething, K. and Steinacker, J. M. (2006). Response and function of skeletal muscle heat shock protein 70. *Front. Biosci.* **11**, 2802-2827.

Loveridge, J. and Craye, G. (1979). Cocoon formation in two species of Southern African frogs. *S. Afr. J. Sci.* **75**, 18-20.

Lutz, G. J., Bremner, S., Lajevardi, N., Lieber, R. L. and Rome, L. C. (1998). Quantitative analysis of muscle fibre type and myosin heavy chain distribution in the frog hindlimb: implications for locomotory design. *J. Muscle Res. Cell. Motil.* **19**, 717-731.

Mantle, B. L., Hudson, N. J., Harper, G. S., Cramp, R. L. and Franklin, C. E. (2009). Skeletal muscle atrophy occurs slowly and selectively during prolonged aestivation in *Cyclorana alboguttata* (Gunther 1867). *J. Exp. Biol.* **212**, 3664-3672.

Mantle, B., Guderley, H., Hudson, N. and Franklin, C. E. (2010). Enzyme activity in the aestivating Green-striped burrowing frog (*Cyclorana alboguttata*). *J. Comp. Physiol.* **180B**, 1033-1043.

McLanahan, L., Shoemaker, V. and Ruibal, R. (1976). The structure and function of the cocoon of a ceratophryd frog. *Copeia* **1976**, 179-186.

Muller, F. L., Song, W., Jang, Y. C., Liu, Y., Sabia, M., Richardson, A. and Van Remmen, H. (2007). Denervation-induced skeletal muscle atrophy is associated with increased mitochondrial ROS production. *Am. J. Physiol.* **293**, R1159-R1168.

Naito, H., Powers, S. K., Demirel, H. A., Sugiura, T., Dodd, S. L. and Aoki, J. (2000). Heat stress attenuates skeletal muscle atrophy in hindlimb-unweighted rats. *J. Appl. Physiol.* **88**, 359-363.

Pinder, A. W., Storey, K. B. and Ultsch, G. R. (1992). Estivation and hibernation. In *Environmental Physiology of the Amphibians* (ed. M. E. Feder and W. W. Burggren), pp. 250-274. Chicago: The University of Chicago Press.

Powers, S. K., Kavazis, A. N. and DeRuisseau, K. C. (2005). Mechanisms of disuse muscle atrophy: role of oxidative stress. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **288**, R337-R344.

Powers, S. K., Kavazis, A. N. and McClung, J. M. (2007). Oxidative stress and disuse muscle atrophy. *J. Appl. Physiol.* **102**, 2389-2397.
Putnam, R. and Bennett, A. (1983). Histochemical, enzymatic and contractile properties of skeletal muscles of three amphibians. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **244**, R558-R567.
Schwinning, S. and Osvaldo, E. S. (2004). Hierarchy of responses to resource pulses in arid and semi-arid ecosystems. *Oecologia* **141**, 211-220.
Seymour, R. S. (1973). Energy metabolism of dormant spadefoot toads (*Scaphiopus*). *Copeia*, 435-445.
Shoemaker, V. H., Balding, D., Ruibal, R. and McClanahan, L. L., Jr (1972). Uricotelism and low evaporative water loss in a South American frog. *Science* **175**, 1018-1020.
Storey, K. B. (1988). Suspended animation - the molecular basis of metabolic depression. *Can. J. Zool.* **66**, 124-132.
Storey, K. B. (1997). Metabolic regulation in mammalian hibernation: enzyme and protein adaptations. *Comp. Biochem. Physiol.* **118A**, 1115-1124.
Storey, K. B. (2002). Life in the slow lane: molecular mechanisms of estivation. *Comp. Biochem. Physiol.* **133A**, 733-754.
Storey, K. B. and Storey, J. M. (1990). Metabolic-rate depression and biochemical adaptation in anaerobiosis, hibernation and estivation. *Q. Rev. Biol.* **65**, 145-174.
Storey, K. B. and Storey, J. M. (2004). Metabolic rate depression in animals: transcriptional and translational controls. *Biol. Rev. Camb. Philos. Soc.* **79**, 207-233.
Symonds, B. L., James, R. S. and Franklin, C. E. (2007). Getting the jump on skeletal muscle disuse atrophy: preservation of contractile performance in aestivating *Cyclorana alboguttata* (Gunther 1867). *J. Exp. Biol.* **210**, 825-835.
Thompson, G., Withers, P., McMaster, K. and Cartledge, V. (2005). Burrows of desert-adapted frogs, *Neobatrachus aquilonius* and *Notaden nichollsi*. *J. R. Soc. West. Aust.* **88**, 17-23.
Tracy, C. R., Reynolds, S. J., McArthur, L., Tracy, C. R. and Christian, K. A. (2007). Ecology of aestivation in a cocoon-forming frog, *Cyclorana australis* (Hylidae). *Copeia*, 901-912.
Turrens, J. F. (2003). Mitochondrial formation of reactive oxygen species. *J. Physiol.* **552**, 335-344.
Van Beurden, E. K. (1980). Energy-metabolism of dormant Australian water-holding frogs (*Cyclorana platycephalus*). *Copeia*, 787-799.
Van Beurden, E. K. (1984). Survival strategies of the Australian water-holding frog, *Cyclorana platycephalus*. In *Arid Australia* (ed. H. G. Cogger and E. E. Cameron), pp. 223-234. Sydney: Australian Museum.
van der Poel, C., Edwards, J. N., Macdonald, W. A. and Stephenson, D. G. (2007). Mitochondrial superoxide production in skeletal muscle fibers of the rat and decreased fiber excitability. *Am. J. Physiol. Cell Physiol.* **292**, C1353-C1360.
Vleck, D. (1987). Measurement of O_2 consumption, CO_2 production, and water-vapor production in a closed system. *J. Appl. Physiol.* **62**, 2103-2106.
Wilson, R. S. and Franklin, C. E. (1999). Thermal acclimation of locomotor performance in tadpoles of the frog *Limnodynastes peronii*. *J. Comp. Physiol. B* **169**, 445-451.
Withers, P. C. (1991). Metabolic depression during aestivation in Australian frogs, *Neobatrachus* and *Cyclorana*. *Aust. J. Zool.* **41**, 467-473.
Withers, P. C. and Thompson, G. G. (2000). Cocoon formation and metabolic depression by the aestivating hylid frogs *Cyclorana australis* and *Cyclorana cultripes* (Amphibia: Hylidae). *J. R. Soc. West. Aust.* **83**, 39-40.