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RESEARCH ARTICLE

Each to their own: skeletal muscles of different function use different biochemical strategies during aestivation at high temperature

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SUMMARY

Preservation of muscle morphology depends on a continuing regulatory balance between molecules that protect and molecules that damage muscle structural integrity. Excessive disruption of the biochemical balance that favours reactive oxygen species (ROS) in disused muscles may lead to oxidative stress, which in turn is associated with increased atrophic or apoptotic signalling and/or oxidative damage to the muscle and thus muscle disuse atrophy. Increases in the rate of oxygen consumption likely increase the overall generation of ROS *in vivo*. Temperature-induced increases in oxygen consumption rate occur in some muscles of ectotherms undergoing prolonged muscular disuse during aestivation. In the green-striped burrowing frog, *Cyclorana alboguttata*, both large jumping and small non-jumping muscles undergo atrophy seemingly commensurate with their rate of oxygen consumption during aestivation. However, because the extent of atrophy in these muscles is not enhanced at higher temperatures, despite a temperature-sensitive rate of oxygen consumption in the jumping muscle, we proposed that muscles are protected by biochemical means that, when mobilised at higher temperatures, inhibit atrophy. We proposed that the biochemical response to temperature would be muscle-specific. We examined the effect of temperature on the antioxidant and heat shock protein systems and determined the extent of oxidative damage to lipids and proteins in two functionally different skeletal muscles, the gastrocnemius (jumping muscle) and the iliofibularis (non-jumping muscle), by aestivating frogs at 24 and 30°C for 6 months. We assayed small molecule antioxidant capacity, mitochondrial and cytosolic superoxide dismutase activities and Hsp70 concentrations to show that protective mechanisms in disused muscles are differentially regulated with respect to both temperature and aestivation. High aestivation temperature results in an antioxidant response in the metabolically temperature-sensitive jumping muscle. We assayed lipid peroxidation and protein oxidation to show that oxidative damage is apparent during aestivation and its pattern is muscle-specific, but unaffected by temperature. Consideration is given to how the complex responses of muscle biochemistry inform the different strategies muscles may use in regulating their oxidative environment during extended disuse and disuse at high temperature.

Key words: disuse, muscle, antioxidant, Hsp70, ROS, oxidative damage, aestivation, temperature.

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INTRODUCTION

Reactive oxygen species (ROS) are biologically important chemicals as they are produced *via* chemical reactions during normal cell metabolism (Liu et al., 2002; Ott et al., 2007; Tahara et al., 2009; Turrens, 2003). Mitochondria produce superoxide (O_2^-) during mitochondrial oxygen consumption (Abele et al., 2002; Murphy, 2009; Tahara et al., 2009), which in turn can trigger a cascade of reactions resulting in the generation of several different reactive species (Cadenas and Davies, 2000; Valentine et al., 1998). ROS are required for signalling purposes (Finkel and Holbrook, 2000; Powers et al., 2010) and proper cellular biochemistry (Kamata and Hirata, 1999; Valentine et al., 1998), so a certain threshold of ROS generation is beneficial to a cell (Hamanaka and Chandel, 2010; Martin and Barrett, 2002). However, at high levels, ROS instigate deleterious cell signalling, disrupting the function of the cell and damaging biological molecules and tissues if left to accumulate (Aruoma, 1998; Benov, 2001; Jones, 2008; Martin and Barrett, 2002; Ott et al., 2007; Valentine et al., 1998). Cellular homeostasis is achieved by molecules that balance the pro-oxidants.

Antioxidants act in a variety of ways to inhibit the action of pro-oxidants by scavenging actual ROS molecules, binding molecules that initiate oxidative chain reactions, and/or acting to break the chain of oxidative reactions (Gutteridge, 1995). Such molecules can include soluble membrane-bound antioxidants (e.g. vitamin E, β-carotene, coenzyme Q), intracellular enzymatic antioxidants (e.g. superoxide dismutases, catalase, glutathione peroxidase) and extracellular antioxidants (e.g. transferrin, albumin, ascorbic acid) (Gutteridge, 1995). Notably, the enzymatic antioxidant superoxide dismutase (SOD) counteracts the superoxide radical produced *via* mitochondrial metabolism, but in doing so produces another ROS, hydrogen peroxide (H_2O_2), which is then counteracted by other antioxidants (Aruoma, 1998). However, disruption of the balance between pro-oxidants and antioxidants can result in a state of oxidative stress.

In addition to antioxidants, heat shock proteins (HSPs) also function during oxidative stress (Kalmar and Greensmith, 2009; Krivoruchko and Storey, 2010; Wallen et al., 1997), facilitating stress sensing, signalling and protein protection (Liu and Steinacker, 2001; Liu et al., 2006; Sørensen et al., 2003). HSPs are regulated in models of muscle disuse (Desplanches et al., 2004; Seo et al., 2006) as well as during dormancy (Lee et al., 2008), and their expression can be tissue and muscle specific (Flanagan et al., 1995;

Locke et al., 1991). Hsp70 has varied cytoprotective functions including molecular chaperoning and refolding of denatured proteins, and inhibition of certain degradative signalling pathways (Feder and Hofmann, 1999; Kalmar and Greensmith, 2009; Muchowski and Wacker, 2005). However, despite the presence of antioxidants and HSPs, the biochemical balance of a tissue can become skewed either *via* antioxidant depletion or *via* increased ROS production (Halliwell and Whiteman, 2004), and subsequently aberrant signalling and damage to cells and tissues can ensue (Benov, 2001; Fulle et al., 2004; Hamanaka and Chandel, 2010; Jones, 2008; Martin and Barrett, 2002; Valentine et al., 1998).

If the ROS generation is sufficiently high, the damage caused by oxidative stress can occur either through direct attack of ROS on biomolecules, or through oxidative-stress-related changes in cellular chemistry (e.g. increased Ca^{2+} levels, protease activation) (Halliwell and Whiteman, 2004). The effects of excessive ROS can include cellular atrophy, apoptosis, and the oxidative modification of lipids, proteins and DNA (Adelman et al., 1988; Gutteridge, 1995; Halliwell and Whiteman, 2004; Hamanaka and Chandel, 2010; McClung et al., 2009; Ott et al., 2007; Powers et al., 2012; Powers et al., 2005; Richter et al., 1988; Valentine et al., 1998). Disruption of redox balance and normal cell signalling processes that result in oxidative stress (Jones, 2008) and tissue damage occur in a variety of conditions including muscle disuse (Costantini et al., 2010; Kavazis et al., 2009; Margaritis et al., 2009; Finkel and Holbrook, 2000; Heise et al., 2003; Lawler et al., 2003; Storey, 1996).

Muscle disuse is associated with atrophic pathways resulting in alterations to muscle phenotype (Zhang et al., 2007), i.e. muscle disuse atrophy (MDA). Notably, ROS can act as upstream triggers for many of those pathways involved in the atrophic remodelling of muscle tissue (e.g. FoxO, NFκβ). Specifically, ROS can act as signalling molecules in situations of muscle disuse such as immobilisation (Kondo et al., 1991; Kondo et al., 1993), limb suspension/unloading (Lawler et al., 2003) and denervation (Muller et al., 2007). The role of ROS and oxidative stress in disuse atrophy is further supported by work showing that mitochondria-targeting antioxidants administered to mice attenuate immobilisation atrophy (Min et al., 2011). However, results from other studies have failed to show any attenuation of disuse atrophy with antioxidant administration (Brocca et al., 2010; Desaphy et al., 2010). This disparity in results is likely due to differences in the specific antioxidants used, the ratio of the magnitude of oxidative stress (threshold level) to the magnitude of the administered antioxidant, the particular muscle tested, different conditions of disuse and combinations thereof.

Dormant animals undergo much less MDA than is seen in artificially immobilised laboratory model organisms (McDonagh et al., 2004; Shavlakadze and Grounds, 2006). Naturally prolonged muscular disuse, as occurs during dormancy in a number of animals, also involves a substantial depression of oxygen consumption and whole-animal metabolic systems with a coordinated reduction in both protein synthesis and protein degradation (Storey and Storey, 1990). Thus, it has been hypothesised that the substantial metabolic depression that accompanies all forms of dormancy reduces overall *in vivo* ROS production and oxidative insult on immobilised muscles, thus limiting MDA (Hudson and Franklin, 2002). Nevertheless, in dormant animals, some atrophy and biomolecular damage still occur.

Lipid peroxidation continues to occur in aestivating freshwater snails, *Biomphalaria tenagophila*, at the same level as in controls (Ferreira et al., 2003), and is elevated during aestivation in spadefoot toad, *Scaphiopus couchii*, skeletal muscle (Grundy and Storey, 1998). The lipid peroxidation in the aestivating toad and snails occurs despite the presence of antioxidant defences. In dormant *S. couchii*, antioxidant defences were tissue-specific and, despite increases in some antioxidants in muscle tissue, the biggest increase in lipid peroxidation levels occurred in the skeletal muscle relative to other organs (Grundy and Storey, 1998). If the low rate of oxygen consumption during aestivation still results in some level of oxidative damage, then ectothermic organisms, such as toads and snails, will presumably experience a greater overall oxidative challenge when temperatures are high.

Recently we have shown in the green-striped burrowing frog, *Cyclorana alboguttata*, that 6 months of aestivation (dry season dormancy) at 30°C significantly increases the rate of oxygen consumption relative to frogs aestivating at 24°C (Young et al., 2011). However, despite the increase in rate of oxygen consumption at 30°C there was no evidence of an increase in the extent of disuse atrophy in the functionally distinct gastrocnemius (a power-producing jumping muscle) or iliofibularis (a small non-jumping muscle) (Young et al., 2012). These findings have led to the hypothesis that protective mechanisms are enhanced during aestivation at high temperature (Young et al., 2012). The present study assessed the mobilisation of protective mechanisms and evidence of oxidative damage in disused muscles of *C. alboguttata* during aestivation at different temperatures. Two functionally and metabolically different muscles (gastrocnemius and iliofibularis) that are resistant to enhanced atrophy at higher temperatures during disuse (Young et al., 2012) were examined. *Cyclorana alboguttata* aestivating for 6 months at either 24 or 30°C were used to assess the levels of small molecule antioxidants (i.e. total antioxidant capacity), the activities of enzymatic antioxidants (mitochondrial and cytoplasmic SOD), levels of HSPs (Hsp70), and markers of oxidative damage (lipid peroxidation and protein carbonylation) in the gastrocnemius and iliofibularis muscles. It was hypothesised that: (1) antioxidants and Hsp70 would be maintained at control levels and/or increased during aestivation, more so at high temperature; (2) the pattern of which protective mechanisms were increased or maintained at controls levels would differ between the two muscles; and (3) oxidative damage would occur during dormancy and to a greater degree in the iliofibularis.

MATERIALS AND METHODS

Experimental animals

Cyclorana alboguttata (Günther 1867) were collected from the districts of Dalby (27.18169 S, 151.26206 E) and Theodore (24.94743 S, 150.07529 E), Queensland, Australia, after heavy rainfall. Frogs were randomly assigned to four treatment groups: controls at 24 and 30°C (N=23 and 18, respectively) and aestivators at 24 and 30°C (N=18 and 21, respectively). Control groups were maintained with weekly feeding at their assigned temperature in thermally controlled facilities for 6 months. Frogs required to aestivate were placed in containers of moist mud and placed at their assigned temperatures in the dark and allowed to burrow and enter aestivation. Aestivating frogs were left for 6 months prior to use.

Aestivating frogs were removed from the dry mud blocks by breaking apart the block at the cracks. All frogs were immediately double-pithed and the gastrocnemius and iliofibularis muscles were dissected out, gently blotted dry and weighed. Muscles were placed in cryotubes, snap-frozen in liquid nitrogen and then stored at –80°C prior to assay. Water content of muscles was not measured but was not expected to change with aestivation or disuse (Bayomy et al., 2002; Cooper, 1972; Mantle et al., 2009). The temperature treatments did not appear to influence overall frog hydration as frogs of both

temperature groups still had obvious bladder water reserves upon extraction from burrows.

All experiments were conducted with the approval of the University of Queensland Animal Welfare Unit and the Queensland Environment Protection Agency.

Total antioxidant capacity

Snap-frozen gastrocnemius and iliofibularis muscle tissues were minced on an ice-cold block and weighed. Minced tissue was diluted 1:10 with ice-cold NP-40 buffer (20 mmol l^{-1} Tris HCl, 137 mmol l^{-1} NaCl, 10% glycerol, 1% nonidet P-40 substitute, 2 mmol l^{-1} EDTA) with protease inhibitors added [P2714, phenylmethylsulfonyl fluoride (PMSF), Sigma-Aldrich Castle Hill, NSW, Australia] and homogenised with an Ika homogeniser (T10-Basic Ultra-Turrax®, IKA, Staufen, Germany). Samples were rocked gently for 30 min on a rocker (Ultra Rocker, Bio-Rad Laboratories, Hercules, CA, USA) and then centrifuged (Allegra 25R, Beckman Coulter, Brea, CA, USA) at 16,000***g*** at 4°C for 20 min. The supernatant was aliquoted in 20 µl volumes and aliquots were stored at –80°C until assaying.

Small molecule and protein antioxidants were assayed using a commercially available kit (K274-100, BioVision, Mountain View, CA, USA) based on a method similar to one previously used for *C. alboguttata* muscles (Mantle et al., 2009), and following the manufacturer's instructions. In brief, a standard curve was produced from prepared Trolox standards (range 0–20 nmol Trolox; R^2=0.9994). Trials indicated an optimal loading volume for samples of 2.5 µl. Samples were loaded in duplicate into individual wells of a 96-well plate and double-distilled H_2O was added to bring the volume to 100 µl. To each well, Cu^{2+} working solution was added. The plate was covered and allowed to incubate at room temperature for 1.5 h before reading the absorbance at 570 nm in a microplate reader (Beckman Coulter DTX 880 Multimode Detector). Absorbance values were used to obtain nmol l^{-1} Trolox equivalents from the standard curve and these were used to calculate the sample antioxidant capacity in nmol μl^{-1}. The total antioxidant capacity (TAC) of each sample was expressed in nmol mg^{-1} tissue.

Superoxide dismutase

Snap-frozen gastrocnemius and iliofibularis muscle tissues were minced on an ice-cold block and weighed. Minced tissue was diluted 1:10 with ice-cold buffer (20 mmol l^{-1} HEPES, 1 mmol l^{-1} EGTA, 210 mmol l^{-1} mannitol, 70 mmol l^{-1} sucrose, pH 7.2) with protease inhibitors added (Sigma-Aldrich P2714, PMSF) and homogenised with an Ika homogeniser (T10-Basic Ultra-Turrax®). Tissue lysates were centrifuged (Beckman-Coulter Allegra 25RA) at 2200***g*** for 5 min at 4°C. The supernatant was transferred to a fresh pre-weighed Eppendorf and centrifuged at 14,300***g*** for 15 min at 4°C. The supernatant (cytosolic fraction) was aliquoted in 25 µl volumes and stored at –80°C. The remaining pellet was weighed and then resuspended 1:10 in cold buffer. The resultant suspension (mitochondrial fraction) was aliquoted in 25 µl volumes and stored at –80°C until assaying.

The cytosolic and mitochondrial activities of the enzymatic antioxidant SOD were assayed (Weydert and Cullen, 2010) using a commercially available kit (product no. 706002, Cayman Chemical Company, Ann Arbor, MI, USA) and following the manufacturer's instructions. In brief, a standard curve was produced from supplied SOD standards (range 0.025–1.0 U ml^{-1} SOD activity; R^2=0.9806). Trials indicated that cytosolic samples required a 15% dilution to fall within the range of the standard curve, while mitochondrial samples were used undiluted. Ten microlitre samples were loaded in duplicate into individual wells of a 96-well plate along with diluted radical detector. Reactions were initiated by adding diluted xanthine oxidase to each well. The plate was shaken briefly to mix reagents, sealed with an adhesive plate cover and then incubated on an orbital shaker (Unimax 1010, Heidolph, Schwabach, Germany) for 20 min at room temperature. Absorbance at 450 nm was read using a microplate reader (Beckman Coulter DTX 880 Multimode Detector). The average absorbance from sample duplicates was used to calculate SOD activity in U ml^{-1} of reaction mix. One 'unit' was regarded as the amount of enzyme required for 50% dismutation of the superoxide radical. SOD activity of the samples were then standardised and expressed as U mg^{-1} tissue. As all samples were handled in a standard manner and SOD inhibitors were absent, SOD activity was representative of the amount of SOD (i.e. levels of the SOD enzyme). Due to logistical limitations, background scavenging of superoxide from non-SOD entities in the biological samples was not measured. As such, these results are more representative of *in vivo* conditions and reflect the superoxide scavenging capacity (SSC) of the muscle tissues, and will be referred to in this manner throughout the Results and Discussion.

Heat shock protein 70

Portions of snap-frozen gastrocnemius and iliofibularis muscles were weighed to the nearest 0.1 mg, individually wrapped in aluminium foil, submerged in liquid nitrogen for 15–20 s and then pulverised on an ice-cold block. Pulverised tissue was removed from the aluminium foil and placed in the bottom of a borosilicate glass micro tissue grinder tube and ground with a pestle. For each tissue portion, 1× extraction reagent (prepared from 5× reagent 80-1581, Enzo Life Sciences, Farmingdale, NY, USA) with added protease inhibitors (Sigma-Aldrich P2714, PMSF) was added in a 1:2 (w/v, g ml^{-1}) ratio, followed by further grinding of tissues. Entire suspensions were transferred to fresh labelled polypropylene tubes and centrifuged (Beckman-Coulter Allegra 25R) at 21,000***g*** for 10 min at 4°C. Supernatants containing the Hsp70 samples were retained in 10 µl aliquots and the remaining pellet was discarded. Aliquots were stored at –80°C until assaying.

HSPs can be detected and quantitated in tissue homogenates using monoclonal antibodies in ELISA assays (Yu et al., 1994). Hsp70 (inducible form) was assayed using a commercially available EIA kit (ADI-EKS-700B, Enzo Life Sciences) and following the manufacturer's instructions. In brief, samples were diluted 1:50 in the supplied diluent. A standard curve was prepared from a serial dilution of the supplied Hsp70 standard (range 0–50 ng ml^{-1}; R^2=0.9999). Standards and diluted samples, in duplicate, were absorbed onto the supplied Hsp70 immunoassay plate for 2 h at room temperature. Wells were washed four times with wash buffer prior to the addition of Hsp70 antibody. Plates were incubated for 1 h at room temperature. Wells were again washed before the addition of Hsp70 conjugate and incubation for an additional hour at room temperature. Wells were again washed and then tetramethylbenzidine substrate solution was added to the wells and the plate was left to incubate for 30 min at room temperature. The development of the colour reaction was stopped by the addition of stop solution. Absorbance was read at 450 nm in a microplate reader (Beckman Coulter DTX 880 Multimode Detector). Sample Hsp70 concentration was calculated from the equation obtained from the standard curve, accounting for the dilution factor (50). Results are expressed as ng mg^{-1} tissue.

Protein oxidation

Snap-frozen gastrocnemius and iliofibularis muscle tissues were minced on an ice-cold block and weighed to the nearest 0.1 mg. Minced tissue was diluted 1:10 with ice-cold buffer (137 mmol l^{-1} NaCl, 2.7 mmol l^{-1} KCl, 4.3 mmol l^{-1} Na_2HPO_4, 1.47 mmol l^{-1} KH_2PO_4) with 5 µl ml^{-1} butylated hydroxytoluene and protease inhibitors were added (Sigma-Aldrich P2714, PMSF), and homogenised with an Ika homogeniser (T10-Basic Ultra-Turrax®). Tissue lysates were centrifuged (Beckman-Coulter Allegra 25R) at 4000 ***g*** for 10 min at 4°C. The supernatant was aliquoted in 20 µl volumes and aliquots were stored at –80°C until assay.

The levels of protein carbonyls were measured using a commercially available kit (STA-310, Cell Biolabs, San Diego, CA, USA) and following the manufacturer's instructions. Protein carbonyls are the most common products of protein oxidation and are chemically stable and serve as oxidative stress markers for most types of ROS (Dalle-Donne et al., 2003; Yan and Sohal, 2002). In brief, protein concentrations of samples were determined using a Qubit™ fluorometer (Q32857, Invitrogen, Mulgrave, VIC, Australia) and a Quant-iT ™ protein assay kit (Invitrogen, Q33211), and samples were then diluted in 0.1 mol l^{-1} phosphate buffered saline (PBS) to 10 µg ml^{-1} protein. A standard curve was produced from 10 µg ml^{-1} oxidised bovine serum albumin (BSA) and 10 µg ml^{-1} reduced BSA for protein carbonyl standards (range 0–7.5 nmol mg^{-1}; R^2=0.9758). Standards and samples were loaded in duplicate into wells of a 96-well protein binding plate and left to adsorb overnight at 4°C. Wells were washed three times with 0.1 mol l^{-1} PBS and then DNPH working solution was added to the wells and the plate was left to incubate in the dark for 45 min. Wells were washed five times with PBS/ethanol (1:1 v/v) and twice with PBS prior to the addition of blocking solution. Plates were left to incubate for 105 min at room temperature on an orbital shaker. Wells were washed three times with wash buffer and then the anti-DNP antibody was added to each well. Plates were left to incubate for 1 h at room temperature on an orbital shaker. Wells were again washed with wash buffer and then a horseradish peroxidase (HRP) conjugated secondary antibody was added to each well and incubated for 1 h at room temperature on an orbital shaker. Wells were washed five times with wash buffer and then substrate solution was added to each well and incubated at room temperature on an orbital shaker under close observation. The reaction was allowed to develop for 4 min before the addition of stop solution to each well. Absorbance at 450 nm was read immediately using a microplate reader (Beckman Coulter DTX 880 Multimode Detector). The average absorbance from sample duplicates was calculated and used in the equation obtained from a polynomial fit of the standard curve to determine the amount of protein carbonyls in nmol mg^{-1} protein.

Lipid peroxidation

Samples were prepared as for protein oxidation above.

The levels of malondialdehyde-adducts (MDA-adducts) in skeletal muscle were measured using a commercially available kit (STA-332, Cell Biolabs) following the manufacturer's instructions. MDA-adducts are an advanced lipid peroxidation end product, formed from a natural by-product of lipid peroxidation, malondialdehyde, which, when bound to protein, form a stable adduct that can be used as a proxy for oxidative stress (Onorato et al., 1998; Requena et al., 1996). In brief, protein concentrations of samples were determined using a Qubit™ fluorometer (Q32857, Invitrogen) and a Quant-iT™ protein assay kit (Q33211, Invitrogen), and the required dilution for each sample was determined in order to prepare samples at 10 µg ml^{-1} protein. A standard curve was produced from a serial dilution of 10 µg ml^{-1} reduced BSA and 0.5 µg ml^{-1} malondialdehyde-BSA (range 0–120 pmol mg^{-1}; R^2=0.9982). Standards and samples were loaded in duplicate into the wells of a 96-well protein binding plate and left to adsorb overnight at 4°C. Wells were washed two times with 0.1 mol l^{-1} PBS and then assay diluents were added to the wells and the plate was left to incubate for 105 min at room temperature on an orbital shaker. Wells were washed three times with wash buffer and then anti-malondialdehyde antibody was added to the wells and incubated for 1 h at room temperature on an orbital shaker. Wells were again washed and secondary antibody-HRP conjugate was added to each well and incubated for an additional 1 h at room temperature on an orbital shaker. Wells were washed five times with wash buffer and then substrate solution was added to each well and the plate was incubated at room temperature on an orbital shaker. The reaction was allowed to develop for 6 min before adding stop solution to each well. The absorbance at 450 nm was read immediately using a microplate reader (Beckman Coulter DTX 880 Multimode Detector). The average absorbance of the sample duplicates was used to determine the amount of MDA adducts. Results are expressed in pmol mg^{-1} protein.

Statistical analyses

Data were assessed *via* a least squares means model considering metabolic state (two levels: control and aestivator), temperature (two levels: 24 and 30°C) and the interaction between these variables. These models were run separately for the gastrocnemius and iliofibularis for total antioxidant capacity, Hsp70, protein oxidation and lipid oxidation data, and run separately for cytosolic and mitochondrial fractions within each muscle for the SOD data. Within-treatment group, between-muscle comparisons were investigated using a least squares means model considering muscle type and the random variable of frog ID, to account for the non-independence of comparing data from muscles obtained from the same source animals. SOD data were similarly assessed but with the additional variable of cellular fraction in the model. Where interaction terms were significant the differences were localised *via* a Tukey's honestly significant difference (HSD) test. In the event that no differences were detected in the overall statistical model, planned pair-wise comparisons of data from frogs aestivating at 24 and 30°C were performed to determine the effects of aestivation temperature on the variables measured. In this case, treatments were compared using ANOVA. In addition, antioxidant data were standardised to muscle-specific rate of oxygen consumption using data reported in Young et al. (Young et al., 2011). The new standard errors were calculated by dividing the original standard errors by their associated means, squaring the result and summing those values for each variable. The square root was taken and then the value was divided by the standardised mean. This presentation of the data provides a perspective of the antioxidants relative to muscle oxygen consumption only and should not be viewed in isolation from the statistically analysed results appearing first.

RESULTS

Total antioxidant capacity

For the gastrocnemius, metabolic state ($F_{1,23}$=4.7916, P=0.039) and the interaction between temperature and metabolic state ($F_{1,23}$=6.1024, P=0.0213) had significant effects on TAC. Specifically, TAC was significantly lower in gastrocnemius muscles from frogs aestivating at 24°C relative to all other groups (Tukey's HSD, P<0.05) and TAC from frogs aestivating at 30°C was at the same level as that of the gastrocnemius of control frogs (Fig. 1A). For the iliofibularis, TAC was significantly lower in muscles from

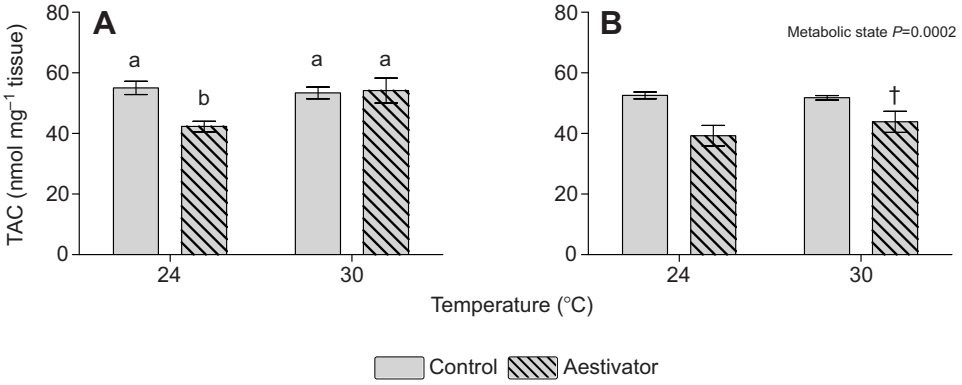

Fig. 1. Total antioxidant capacity (TAC) of (A) gastrocnemius and (B) iliofibularis muscles in *Cyclorana alboguttata*. Data are expressed as means ± s.e.m. Significant parameters from the analysis are inset in B (see Materials and methods for models). Letters detail the results of *post hoc* analysis on a significant interaction term. Bars with different letters are significantly different. † indicates a significant difference of the iliofibularis value from the gastrocnemius value for the associated treatment group as determined by least squares means model. Sample sizes for all bars are N=7 except the 24°C aestivator groups, where N=6.

aestivating frogs relative to control frogs ($F_{1,23}$=18.879, P=0.0002; Fig. 1B) irrespective of treatment temperature.

Between all four treatment groups, there was no significant difference in the TAC of gastrocnemius and iliofibularis muscles except for tissues from the 30°C aestivator group, where the TAC was significantly higher in the gastrocnemius muscle than in the iliofibularis muscle ($F_{1,6}$=24.3782, P=0.0026).

When standardised to the oxygen consumption rate of the muscles, both the gastrocnemius and the iliofibularis from aestivating frogs had greater TAC than those from control frogs. Standardising TAC to the oxygen consumption rate of the gastrocnemius from aestivating frogs revealed there was greater TAC during aestivation at 24°C than at 30°C (Fig. 2A). However, the iliofibularis from aestivating frogs showed similar TAC (per unit metabolic rate) between temperatures (Fig. 2B).

Superoxide scavenging capacity

For the gastrocnemius, there was a significant interaction between metabolic state and temperature on cytosolic SOD/SSC ($F_{1,24}$=7.5541, P=0.0112) yet the *post hoc* analysis failed to identify any family-wise differences between groups. Comparing between aestivating groups, gastrocnemius cytosolic SOD/SSC was significantly higher in frogs aestivating at 30°C compared with those aestivating at 24°C ($F_{1,12}$=5.8228, P=0.0327; Fig. 3A). Mitochondrial SOD/SSC in the gastrocnemius was significantly higher in aestivating frogs than in control frogs ($F_{1,24}$=15.8068, P=0.0006; Fig. 3C). Comparing between aestivating groups, gastrocnemius mitochondrial SOD/SSC was significantly higher in frogs aestivating at 30°C compared with those aestivating at 24°C ($F_{1,12}$=5.5878, P=0.0358; Fig. 3C).

For the iliofibularis, cytosolic SOD/SSC was higher in aestivating frogs compared with controls ($F_{1,24}$=20.877, P=0.0001) and also higher in both aestivating and control frogs at 30°C compared with at 24°C ($F_{1,24}$=9.6521, P=0.0048; Fig. 3B). Mitochondrial SOD/SSC was higher in the iliofibularis from aestivating frogs than in control frogs irrespective of temperature ($F_{1,22}$=9.0735, P=0.0064; Fig. 3D).

In all treatment groups, muscle type, the cellular fraction examined and the interaction between muscle and cellular fraction had a significant effect on SOD/SSC (all P<0.03), with the cytosolic fractions showing significantly more SOD/SSC than the mitochondrial fractions (P<0.05). In addition, the gastrocnemius showed significantly more cytosolic SOD/SSC than the iliofibularis (P<0.05; Fig. 3B). Mitochondrial SOD/SSC was not different between the two muscles.

When standardised to oxygen consumption rate of the appropriate muscle type, cytosolic and mitochondrial SOD/SSC were always greater in the gastrocnemius and iliofibularis from aestivating frogs than in control frogs. In the gastrocnemius from aestivating frogs, both cytosolic and mitochondrial SOD/SSC (per unit metabolic rate) were greater at 24°C compared with 30°C (Fig. 4A). In the iliofibularis from aestivating frogs, cytosolic SOD/SSC was greater in iliofibularis from frogs aestivating at 30°C compared with 24°C, whereas mitochondrial SOD/SSC was similar between the two temperatures (Fig. 4B).

Heat shock protein 70

For the gastrocnemius, Hsp70 levels were higher from aestivating frogs than from control frogs ($F_{1,23}$=5.4635, P=0.0285; Fig. 5A). In contrast, for the iliofibularis, Hsp70 levels were significantly lower in the iliofibularis from aestivating frogs compared with control frogs ($F_{1,24}$=40.9058, P<0.0001; Fig. 5B). Within the aestivating groups, iliofibularis Hsp70 levels were significantly lower in frogs aestivating at 30°C compared with frogs aestivating at 24°C ($F_{1,12}$=4.9130, P=0.0467). Because of to the small magnitude of the

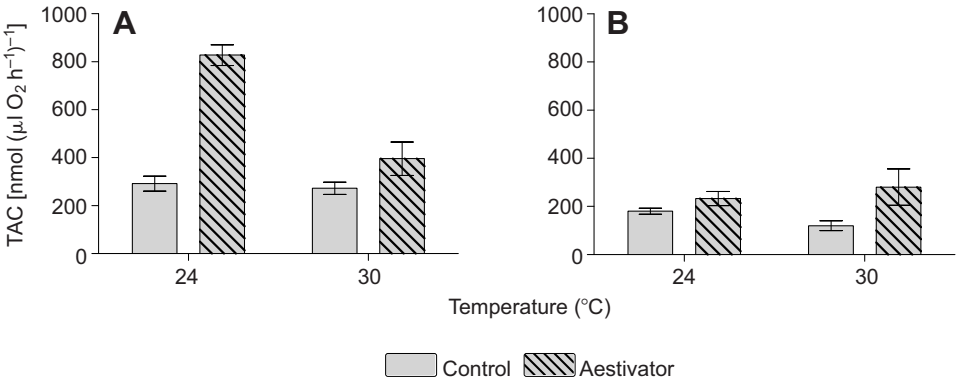

Fig. 2. Total antioxidant capacity (TAC) standardised to muscle rate of oxygen consumption of (A) gastrocnemius and (B) iliofibularis muscles in *Cyclorana alboguttata*. Data are expressed as means ± s.e.m. As the standardised means and standardised errors were calculated from the original means and errors of the data in Fig. 1 and muscle oxygen consumption data from Young et al. (Young et al., 2011), statistical significance cannot be indicated. See Materials and methods for calculation.

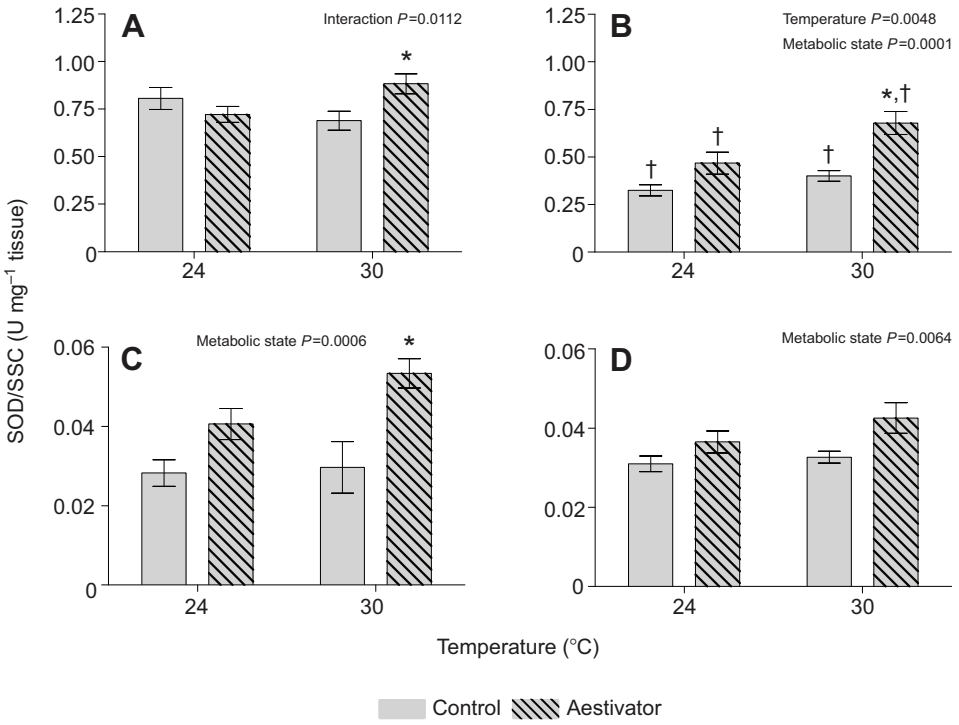

Fig. 3. Superoxide scavenging capacity (SOD/SSC) in the (A) gastrocnemius cytosolic fraction, (B) iliofibularis cytosolic fraction, (C) gastrocnemius mitochondrial fraction and (D) iliofibularis mitochondrial fraction in *Cyclorana alboguttata*. Data are expressed as means ± s.e.m. Significant parameters from the analysis are inset on each graph (see Materials and methods for models). † indicates a significant difference of the iliofibularis value from the gastrocnemius value for the associated treatment group as determined by the least squares means model (within the cellular fraction). * indicates a significant difference between 30°C aestivator and 24°C aestivator values as determined by planned comparison ANOVA. Sample sizes for all bars is *N*=7 except in D, where the 24°C and 30°C aestivator bars are *N*=6.

changes (3–5%), effect sizes were calculated according to Coe (Coe, 2002). For changes in response to aestivation, effect sizes for gastrocnemius at 24 and 30°C were both 0.9. The effect size for iliofibularis at 24°C was 1.7 and at 30°C the effect size was 3.5. The effect size for the decrease in Hsp70 with increased temperature for iliofibularis from aestivators was 1.2. In all treatment groups, Hsp70 was significantly lower in the gastrocnemius than in the iliofibularis (P<0.0001).

Protein oxidation

For the gastrocnemius, protein carbonyl levels were unaffected by temperature or metabolic state, meaning that all treatment groups showed similar levels of protein carbonylation (Fig. 6A). For the iliofibularis, protein carbonyl levels were significantly higher in the iliofibularis from aestivating frogs compared with control frogs ($F_{1,23}$=35.9678, P<0.0001; Fig. 6B). Within control treatments there was no difference in the level of protein carbonyls between the gastrocnemius and iliofibularis at either temperature. However, in aestivating frogs, protein carbonyl levels in the gastrocnemius were significantly lower than those in the iliofibularis at both 24°C ($F_{1,5}$=25.3643, P=0.0037) and 30°C ($F_{1,4}$=11.0227, P=0.0294).

Lipid oxidation

For the gastrocnemius, lipid peroxidation levels were unaffected by temperature or metabolic state, meaning that all treatment groups showed similar levels of lipid peroxidation (Fig. 7A). In contrast, for the iliofibularis, lipid peroxidation was significantly lower in aestivating frogs relative to control frogs ($F_{1,21}$=8.0293, P=0.0099; Fig. 7B). Within all treatment groups, the level of lipid peroxidation was significantly higher in gastrocnemius muscles than in the iliofibularis muscles (all P<0.004).

DISCUSSION

There are many biochemical pathways and regulatory mechanisms in play during hypometabolism, muscular disuse, atrophy and oxidative stress. The combination of these factors during aestivation in an ectothermic frog presents a unique model for investigating the complex interplay between these factors and the associated biochemical mechanisms. Our data suggest that the gastrocnemius and iliofibularis muscles, and presumably other skeletal muscles of *C. alboguttata*, deal with similar challenges (metabolic, thermal) in different ways. Significant differences were apparent in some protective/reparative systems in response to higher temperature, and significant differences in protective mechanisms and oxidative damage were also apparent between the two muscle types in response to aestivation (Table 1).

Protective mechanisms

Antioxidants

Aestivation temperature significantly influenced regulation of cytoprotective mechanisms but did so differently in gastrocnemius and iliofibularis muscle of *C. alboguttata*. Significant elevation of both mitochondrial and cytosolic SOD/SSC in the gastrocnemius of frogs aestivating at 30°C compared with 24°C is consistent with the elevated rate of oxygen consumption of the gastrocnemius during aestivation at 30°C (Young et al., 2011). The concurrent increase in gastrocnemius TAC during aestivation at 30°C suggests that the overall production of ROS in the muscle may have been higher, enhancing the requirement of the gastrocnemius for small molecule and protein antioxidants. The lack of a temperature effect on iliofibularis mitochondrial SOD/SSC is consistent with the lack of a temperature effect on rate of oxygen consumption in the iliofibularis (Young et al., 2011).

The upregulation of some antioxidant defences in response to higher temperature in aestivation is consistent with the hypothesis that higher temperatures may induce a more pro-oxidant cellular environment. This is especially so in the case of the gastrocnemius, where the rate of oxygen consumption was increased at the higher aestivation temperature (Young et al., 2011). However, the iliofibularis had a higher mass-specific rate of oxygen consumption than the

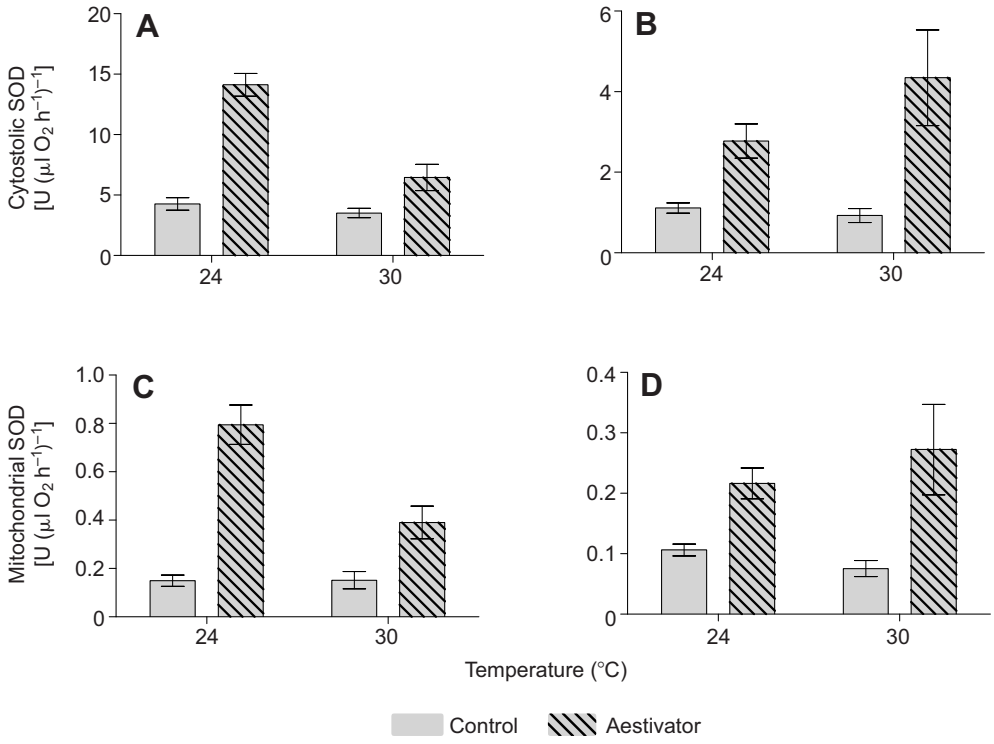

Fig. 4. Superoxide scavenging capacity standardised to muscle rate of oxygen consumption for the (A) gastrocnemius cytosolic fraction, (B) iliofibularis cytosolic fraction, (C) gastrocnemius mitochondrial fraction and (D) iliofibularis mitochondrial fraction in *Cyclorana alboguttata*. Data are expressed as means ± s.e.m. Note the necessary difference in scale. As the standardised means and standardised errors were calculated from the original means and errors of the data in Fig. 3 and muscle oxygen consumption data from Young et al. (Young et al., 2011), statistical significance cannot be indicated. See Materials and methods for calculation.

gastrocnemius (Young et al., 2011) and could therefore be considered to experience greater oxidative insult, which is consistent with the greater atrophy in the iliofibularis compared with the gastrocnemius (Young et al., 2012). Muscle-specific antioxidant scavenging capacity has also been reported for rats (Masuda et al., 2003).

Two main strategies regarding antioxidant action in aestivation have been proposed: (1) antioxidant defences track metabolic rate and potentially directly respond to an imposed stressor, and (2) high levels of antioxidants are maintained in preparation for arousal events (‘pre-emptive’ regulation) (Ferreira-Cravo et al., 2010; Storey, 1996). The latter is based on the theory that the increase in metabolic processes that occurs with arousal results in elevated oxygen concentration and sufficient ROS generation to overwhelm antioxidant defences and be damaging. Thus, an increase in oxygen consumption during aestivation, such as that induced by temperature, may, if large enough, also present an oxidative insult sufficient to be damaging, unless pre-emptive protective strategies are used. Pre-emptive regulation of antioxidants may manifest as either an upregulation of antioxidants or their maintenance at control levels, despite a lowered (presumed) ROS insult, such that defences are prepared for a later ROS insult. The differential regulation of TAC and SOD/SSC with respect to both aestivation and temperature suggests that strategies of tracking metabolic rate and pre-emptive regulation may both be in operation in *C. alboguttata*. For example, iliofibularis TAC shows the same changes in response to aestivation and temperature as iliofibularis rate of oxygen consumption (Young et al., 2011). All antioxidants measured here for the gastrocnemius of aestivators increase along with the increased rate of oxygen

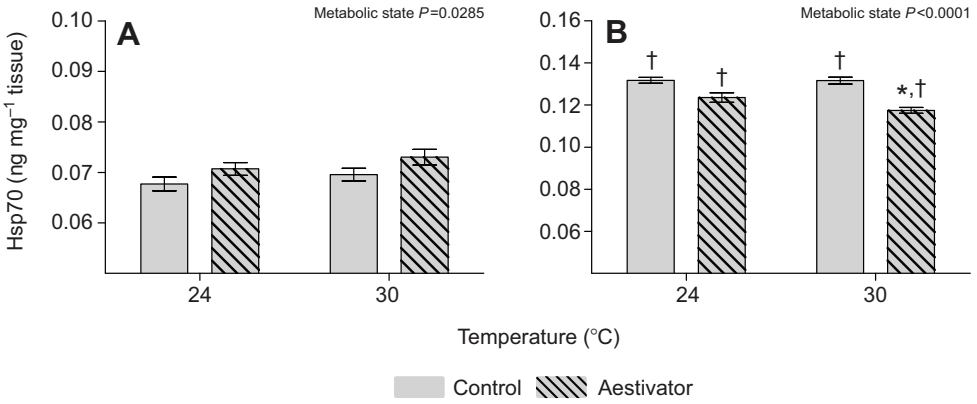

Fig. 5. Heat shock protein 70 (Hsp70) of (A) gastrocnemius and (B) iliofibularis muscles in *Cyclorana alboguttata*. Data are expressed as means ± s.e.m. Significant parameters from the analysis are inset on each graph (see text for models). † indicates a significant difference of the iliofibularis value from the gastrocnemius value for the associated treatment group as determined by the least squares means model. * indicates a significant difference between 30°C aestivator and 24°C aestivator values as determined by planned comparison ANOVA. Note the difference in scale on the *y*-axes. Sample sizes for all bars are *N*=7 except for the 24°C control bar in A, where *N*=6.

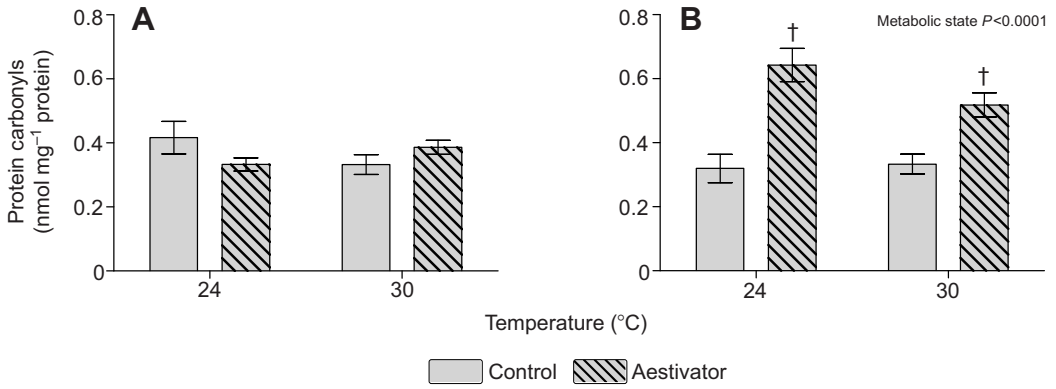

Fig. 6. Protein carbonyl content of (A) gastrocnemius and (B) iliofibularis muscles in *Cyclorana alboguttata*. Data are expressed as means ± s.e.m. Significant parameters from the analysis are inset on each graph (see Materials and methods for models). † indicates a significant difference of the iliofibularis value from the gastrocnemius value for the associated treatment group as determined by the least squares means model. For A, *N*=[7, 6, 7, 6]; for B, *N*=[7, 7, 7, 6].

consumption of the muscle at the higher temperature. Our data suggest that pre-emptive regulation may occur for iliofibularis SOD/SSC in aestivation and with higher temperature, and for gastrocnemius mitochondrial SOD/SSC with aestivation. Of course, these patterns of regulation cannot be construed as the entire 'strategy' used by the muscles because other antioxidants, not measured here, will also be regulated. However, the changes in specifically mitochondrial SOD/SSC shown in this study may in part account for the lack of enhanced atrophy at 30°C, as mitochondrial-targeted antioxidants have been shown to attenuate immobilisation atrophy in mice (Min et al., 2011). Interestingly, it is possible that the rise in mitochondrial SOD/SSC is part of regulating the muscle cells in a quiescent state by producing increased hydrogen peroxide, promoting cellular quiescence (Sarsour et al., 2008).

The rising SOD/SSC level specifically in the cytosolic fraction is curious if the production of ROS is related primarily to rate of mitochondrial oxygen consumption. It is possible that oxygen consumption of non-mitochondrial sources, such as NADPH-oxide synthase, is responsible, perhaps more so in the case of the iliofibularis than the gastrocnemius. Alternatively, a mechanism for increased superoxide of mitochondrial origin in the cytosol is provided by the passage of inter-membrane space superoxide *via* voltage-dependent anion channels in the outer mitochondrial membrane (Han et al., 2003), potentially as part of intracellular signalling (Hamanaka and Chandel, 2010; Murphy, 2009; Murphy et al., 2011). It has been reported that superoxide can be released from the mitochondria into the cytosol at a rate of ~0.041 nmol min^{-1} mg^{-1} protein in preparations of rat heart mitochondria, although it is possible this rate is an underestimate (Han et al., 2003). In the case of disuse during dormancy, the oxidant environment of a muscle from an aestivator will be a product of the relative magnitudes of changes in antioxidants levels and rate of oxygen consumption of the tissue.

The substantial reduction in rate of oxygen consumption of muscles during aestivation meant that antioxidant levels per unit muscle metabolic rate (i.e. antioxidants relative to oxidative insult) were always higher in muscle from aestivating frogs compared with controls, irrespective of upregulation or downregulation of the antioxidants. Despite this beneficial antioxidant environment, both the iliofibularis and the gastrocnemius undergo some atrophy, although the extent is not greater at 30°C compared with 24°C (Young et al., 2012). The gastrocnemius antioxidant per metabolic rate environment in aestivation was less at 30°C than at 24°C. This indicates that the upregulation of gastrocnemius TAC and SOD/SSC defences in response to high aestivation temperature were not of a large enough magnitude, relative to the magnitude of the increase in rate of oxygen consumption (i.e. increased oxidative insult), to maintain the same TAC/SOD environment as at 24°C. However, this change does not result in greater oxidative damage. It is possible that the composition of the antioxidant 'cocktail' in aestivating gastrocnemius muscle is altered at different temperatures, and measures of other antioxidants would clarify whether antioxidants not measured here were increased in the gastrocnemius at 30°C. Interestingly, thiols are a major determinant of the antioxidant capacity (i.e. TAC) of tissue homogenates (Balcerczyk and Bartosz, 2003) and redox-sensitive thiol disruption is posited to be more common than, and occur prior to, oxidative damage to macromolecules (Dalle-Donne et al., 2001; Jones, 2008). Thus, increased TAC at 30°C in aestivating gastrocnemius is potentially indicative of an intermediate threshold of ROS production despite the presence of lipid and protein oxidation. The lack of a temperature effect on iliofibularis rate of oxygen consumption, TAC and mitochondrial SOD/SSC in aestivation meant that these antioxidant defences per unit metabolic rate were equivalent between the two aestivation temperatures. Consequently, the higher level of iliofibularis cytosolic SOD/SSC per unit metabolic rate during aestivation at 30°C was achieved by upregulating SOD/SSC in aestivation but to a greater magnitude at 30°C than at 24°C. The exact way the changes to antioxidant environments of the gastrocnemius and iliofibularis relates to the patterns of oxidative damage reported in these muscles is uncertain and suggests that other protective mechanisms may also be at play in a muscle-specific fashion.

Hsp70

As our method tested for the inducible form of Hsp70, our data show that inducible Hsp70 was constitutively expressed in *C. alboguttata* gastrocnemius and iliofibularis muscle. The changes in Hsp70 with aestivation and, in the case of the iliofibularis, with temperature were small (~5%), yet, according to a percentile interpretation (Coe, 2002), the effect sizes (0.9–3.5) indicate we can be confident that these changes are meaningful. In exactly what biological way these changes manifest remains unclear. The small magnitude of change in *C. alboguttata* suggests that Hsp70 expression levels are tightly regulated. The decrease in the iliofibularis Hsp70 with aestivation may also be a response to constant temperature conditions. Hsp70 can be downregulated with prolonged exposure to a mild stressor (Abravaya et al., 1991) and therefore it is possible that the Hsp70 results recorded here are a temporal snapshot of Hsp70 regulation given that measurements were taken only once at 6 months of aestivation. Because *C. alboguttata* Hsp70 was regulated more so in response to aestivation than in response to temperature, it is likely that the regulation of constitutive Hsp70 levels was effective irrespective of temperature. At both temperatures iliofibularis Hsp70 was reduced in aestivation

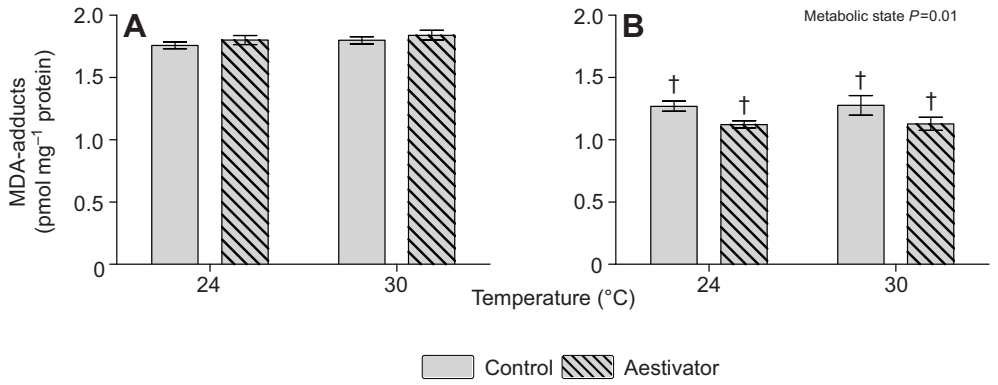

Fig. 7. Malondialdehyde-adduct (MDA-adduct) content of (A) gastrocnemius and (B) iliofibularis muscles in *Cyclorana alboguttata*. Data are expressed as means ± s.e.m. Significant parameters from the analysis are inset on each graph (see Materials and methods for models). † indicates a significant difference of the iliofibularis value from the gastrocnemius value for the associated treatment group as determined by the least squares means model. For A, *N*=7; for B, *N*=[7, 7, 6, 5].

compared with controls, which is consistent with previous results in hind-limb suspended mice, where Hsp70 and other HSPs were downregulated in the oxidative soleus muscle (Brocca et al., 2010).

Unlike the iliofibularis, Hsp70 levels in the gastrocnemius increased during aestivation, suggesting that the gastrocnemius may have had an increased requirement for Hsp70 during aestivation. Increased expression of HSP in the muscles of dormant animals has been reported in hibernating bats (*Murina leucogaster*, 1.7-fold) (Lee et al., 2008) and hibernating 13-lined ground squirrels (*Spermophilus tridecemlineatus*, twofold to threefold) (Carey et al., 1999). Hsp70 regulation in opposite directions in different muscles during dormancy suggests that different muscles have different requirements for Hsp70 and/or that the muscles are subject to different signalling environments. Significant muscle-specific differences in constitutive levels of HSP expression are also found between unstressed rat muscles (Locke et al., 1991). Hsp70 expression levels in skeletal muscle are known to be dependent on the intensity of the stressor (Liu et al., 2006), potentially indicating that the iliofibularis of *C. alboguttata* experiences a greater oxidative insult than the gastrocnemius. However, given the constitutive expression of Hsp70 in both muscles, a dose-dependent response of Hsp70 to a stressor may be less coupled.

Different oxidants can regulate the heat shock response to different degrees (Wallen et al., 1997). Thus, the differential regulation of the antioxidants and oxidative damage patterns between the iliofibularis and gastrocnemius of aestivating *C. alboguttata* is likely to subject the muscles to differing oxidant environments, potentially explaining muscle-specific Hsp70 regulation. Alternatively, the regulation of Hsp70 in *C. alboguttata* skeletal muscles could be associated with some other stimulus, such as local hypoxia. In submerged overwintering *Rana temporaria*, 1 month of hypoxia resulted in a significant increase in Hsp70 in heart muscle but Hsp70 levels returned to control values within 4 months of hypoxia, suggesting that the earlier stages of overwintering may be more physiologically stressful than the later stages (Currie and Boutilier, 2001). If such were true for *C. alboguttata*, a 'peak' in Hsp70 regulation may have been missed because measurements were only taken after 6 months of aestivation. Inducible HSPs respond to a number of stressors (e.g. temperature, oxygen levels, oxidative stress, cellular energy depletion, toxic substances) if those stressors are sufficiently high and the threshold for induction is generally correlated with the physiological stress an organism experiences naturally (Feder and Hofmann, 1999). The sufficient stressor operating on *C. alboguttata* gastrocnemius muscle appears to be associated more with aestivation than with temperature in this study.

The exact proximal triggers of Hsp70 regulation and Hsp70's role(s) during muscle disuse that occurs with aestivation in *C. alboguttata* are indistinct. However, Hsp70 regulation may in part be linked to antioxidant regulation. For example, administration of exogenous antioxidants to hind-limb unloaded mice and rats correlates with an increase in Hsp70 expression in the oxidative soleus muscle (Brocca et al., 2010; Servais et al., 2007). However, this is not always the case (Desplanches et al., 2004; Selsby and Dodd, 2005). Constitutive Hsp70 expression and reduced oxygen consumption in aestivation suggests that metabolic state-regulated changes in Hsp70 are perhaps not a response to altered oxidative stress *per se*, but possibly a pre-emptive yet muscle-specific strategy. In such a case, the increase in gastrocnemius Hsp70 with aestivation may be part of a preferential protection of a large jumping muscle over a smaller non-jumping muscle.

Table 1. Summary of the data

		Protective mechanisms				Oxidative damage	
Comparison	Group/Muscle	Antioxidant capacity	Cytosolic SOD/SSC	Mitochondria l SOD/SSC	Hsp70	Lipid peroxidation	Protein carbonylation
(1) Within aestivation, with higher temperature	Gastrocnemius	↑	↑	↑	—	—	—
	Iliofibularis	—	↑	—	↓	—	—
(2) With transition from non-aestivating to aestivating	Gastrocnemius	24°C ↓, 30°C —	—	↑	↑	—	—
	Iliofibularis	↓	↑	↑	↓	↓	↑
(3) Within aestivation and temperature (between muscles)	24°C aestivating	=	G>I	=	I>G	G>I	I>G
	30°C aestivating	G>I	G>I	=	I>G	G>I	I>G

SOD, superoxide dismutase; SSC, superoxide scavening capacity.

Results are divided into three main comparisons of interest. For comparisons 1 and 2, upwards arrows indicate an increase in the parameter, downwards arrows indicate a decrease in the parameter, and horizontal lines indicate no change. For comparison 3, 'I' refers to the iliofibularis, 'G' refers to the gastrocnemius, and '=' indicates levels of parameter were not different between the muscles.

Constitutive expression of Hsp70 may protect against expected accumulation of oxidative stress over prolonged aestivation and against the oxidative insult upon arousal, and presumably further helps *C. alboguttata* to 'absorb' temperature fluctuations during aestivation. Constitutive expression of HSPs are a strategy common to amphibians (Chapovetsky and Katz, 2006) regardless of varying physiological tolerance to environmental stressors, and may be a strategy common to animals that undergo dormancy (Carey et al., 1999; Storey and Storey, 2011). Other HSPs with well-defined roles in muscle morphology and function such as Hsp27 (Folkesson et al., 2008; Sharp et al., 2006) and Hsc70 (heat shock *cognate* 70) *via* interactions with thiols (Hoppe et al., 2004) are also likely to be involved. Therefore, a certain level of oxidants in aestivating muscle may improve the protection of the muscle tissue *via* regulation of HSPs.

Oxidative damage

Lipid peroxidation

The presence of muscle protective mechanisms does not entirely prevent oxidative damage to macromolecules. Lipid peroxidation cannot be caused by superoxide or hydrogen peroxide but can be caused by the hydroxyl radical (Gutteridge, 1995), indicating that production of superoxide escapes SOD defences and may contribute to the production of the hydroxyl radical, which in turn must evade the TAC defences. Lipid peroxidation levels occurring in the muscles of aestivating *C. alboguttata* were not altered by the temperature at which the frogs aestivated. In the iliofibularis this is potentially somewhat related to the temperature-insensitive rate of oxygen consumption (Young et al., 2011) and the equivalent levels of antioxidants relative to oxidative insult at both 24 and 30°C, though this assumes that oxidant production from non-mitochondrial respiration sources is not altered. In the gastrocnemius of aestivating frogs, the levels of lipid peroxidation were also equivalent between 24 and 30°C despite an elevated rate of muscle oxygen consumption during aestivation at 30°C (Young et al., 2011). However, unlike in the iliofibularis, the (measured) antioxidants were higher at 30°C and possibly acted to reduce the oxidative insult sufficiently at 30°C so that oxidative damage to lipids did not exceed control levels. This is consistent with other studies reporting that administration of vitamin E (a small molecule antioxidant) results in decreased lipid peroxidation in skeletal muscle (Kondo et al., 1991; Servais et al., 2007). The reduced iliofibularis lipid peroxidation in aestivation when TAC is also reduced, and no reduction of gastrocnemius lipid peroxidation in aestivation when TAC is reduced at 24°C or increased at 30°C, suggests a different suite of *in vivo* conditions between the two muscles, such as muscle-specific regulation of other antioxidants, mitochondrial properties, degree of coupling of ROS formation to oxygen consumption and differences in the signalling requirements and metabolic program changes of the muscles (Anderson and Neufer, 2006; Brocca et al., 2010).

Because products of lipid peroxidation can form part of a negative feedback loop in the muscle and act to induce a mild mitochondrial uncoupling and reduce ROS (Jastroch et al., 2010), some degree of lipid peroxidation may actually be beneficial. However, if such a feedback loop were in operation, the degree to which this would reduce ROS *via* mitochondrial uncoupling is also uncertain because during aestivation *C. alboguttata* are reported to increase mitochondrial coupling (Kayes et al., 2009). The accumulation of lipid peroxidation during aestivation despite antioxidant regulation is consistent with aestivating spadefoot toads (Grundy and Storey, 1998), land snails (Hermes-Lima and Storey, 1995), freshwater snails (Ferreira et al., 2003) and hibernating little susliks (L'vova and Gasangadzhieva, 2003). Lipid damage accumulates in aestivating spadefoot toads in liver, heart, gut and kidney, and the highest accumulation of damage in aestivation, relative to non-aestivating toads, is in the skeletal muscle (2.7-fold higher) (Grundy and Storey, 1998), though overall levels of peroxidation are relatively low. For example, in thigh muscles of healthy humans (<40 years old), lipid peroxidation is reported at 30 pmol mg^{-1} protein (Mecocci et al., 1999), so in aestivating *C. alboguttata* peroxidation is relatively low at 1.8 pmol mg^{-1} protein in the gastrocnemius and 1.1 pmol mg^{-1} protein in the iliofibularis.

Despite significantly more lipid peroxidation occurring in the gastrocnemius relative to iliofibularis, levels were consistent between all four treatment groups, which therefore suggests that the overall level of lipid peroxidation was physiologically acceptable, irrespective of metabolic state. A high threshold of (the appropriate) ROS is required for any oxidative modifications to be detrimental to the biology of the organism (Hamanaka and Chandel, 2010). Detrimental or not, lipid peroxidation in *C. alboguttata* muscles during aestivation may indicate something about the relative hydroxyl radical environment between the muscles. However, oxidative damage, as indicated by protein carbonylation, showed a different pattern.

Protein carbonylation

Temperature did not influence the level of protein carbonylation occurring in either the gastrocnemius or the iliofibularis during aestivation. In the iliofibularis, this may be attributed to the insensitivity of the aestivating rate of oxygen consumption to temperature (Young et al., 2011) and therefore the lack of a change in ROS production with temperature. However, the lower rate of oxygen consumption in aestivation (and presumably lower overall ROS) does not reconcile with protein carbonylation in response to aestivation for the iliofibularis muscle, where carbonylation significantly increases. Increased carbonylation occurred despite the iliofibularis having greater antioxidants (those measured here) relative to the presumed oxidative insult from oxygen consumption during aestivation compared with controls. Similarly, in the gastrocnemius the lack of temperature effect on the level of protein carbonylation may result from the antioxidant environment during aestivation at 30°C being sufficient to counteract the temperature-induced rise in oxygen consumption and presumed overall ROS generation. Additionally, the significant increase in Hsp70 in aestivating gastrocnemius relative to controls may be associated with constraining protein carbonylation at control levels (Fredriksson et al., 2005). Alternatively, Hsp70's role may be in protecting the cellular proteome because of the fact that protein carbonylation continues at control levels in aestivation (Krivoruchko and Storey, 2010).

Hsp70 is involved in the identification and degradation of carbonylated proteins (Kalmar and Greensmith, 2009). Therefore, the high level of Hsp70 in the iliofibularis of *C. alboguttata* relative to the gastrocnemius suggests a link between Hsp70 function and protein carbonylation in the iliofibularis. Moreover, molecular chaperones can also be targets of oxidative damage (Dukan and Nyström, 1999; Oikawa et al., 2009; Tamarit et al., 1998). It is conceivable that the high Hsp70 levels in the iliofibularis during aestivation could even contribute to the high levels of carbonylated proteins in the iliofibularis. However, it is more likely that Hsp70 functions in a cytoprotective manner given that aestivation is an adaptive strategy for prolonged survival.

Despite the coordinated downregulation of biochemical processes in aestivation (Bishop and Brand, 2000; Cowan et al., 2000; Cowan and Storey, 1999; Storey and Storey, 1990), muscle-specific regulation of metabolic enzymes can maintain some muscles in a 'primed' state for arousal (e.g. powerful locomotory muscles) and others in a less primed state (Mantle et al., 2010). Maintaining a muscle in a more 'primed' state when substrates are likely at diminished levels may make its enzymatic pool more susceptible to oxidation (Nyström, 2005). Thus, muscle-specific enzyme regulation may contribute to muscle-specific patterns of protein oxidation. Despite the patterns of protein carbonyl levels with aestivation and muscle type, it is possible that all of the protein carbonyl measurements are at an 'acceptable' biological level. The exact identity of the carbonylated proteins is unknown so it is possible that no one given type of protein is damaged in large enough amounts to disrupt biological function. As a guide, in wood frogs, *Rana sylvatica*, protein carbonyls in muscle tissue of control frogs at 5°C is 0.422 nmol mg^{-1} protein (reported as 422 pmol mg^{-1} protein) rising to 0.601 nmol mg^{-1} protein in winter frozen frogs (reported as 601 pmol mg^{-1} protein) (Woods and Storey, 2006). The highest protein carbonyl content in *C. alboguttata* was the significant increase in carbonyls in the iliofibularis of aestivators at ~0.6 nmol mg^{-1} protein, while the gastrocnemius of aestivators along with control muscles showed protein carbonyl levels of ~0.4 nmol mg^{-1} protein or less, highly consistent with the wood frog.

With the protracted duration of aestivation in *C. alboguttata*, targeted protein carbonylation may play a role in the acquisition of muscle protein for metabolic fuel (Grably and Piery, 1981) or urea synthesis (Withers and Guppy, 1996). Therefore, the significantly higher protein carbonylation during aestivation in the iliofibularis relative to the gastrocnemius might reflect selective protein acquisition from non-jumping muscles over a power-producing muscle required for immediate post-aestivation activity. Recent thinking views the threshold of ROS/oxidative stress that results in detrimental oxidative damage to macromolecules to be higher than the threshold for signalling apoptosis (Hamanaka and Chandel, 2010). Thus, protein carbonylation in the iliofibularis during aestivation might indicate that there was an increase in oxidative stress. However this does not reconcile with the muscle's metabolic depression with aestivation if the overall ROS production is reduced in aestivation, as assumed, or the seemingly low overall levels of protein oxidation. In any case, iliofibularis protein oxidation increases in aestivation and is higher than in gastrocnemius, seemingly consistent with the greater muscle disuse atrophy in iliofibularis than gastrocnemius (Young et al., 2012). More direct studies of ROS, a range of antioxidant systems and other regulatory processes should help resolve the biological relevance of the protein carbonyl results.

Concluding remarks

It is clear that the iliofibularis and gastrocnemius of *C. alboguttata* undergo quantitatively different oxidative 'experiences' during both aestivation in general and aestivation at elevated temperatures (Table 1). The need to conserve energy during aestivation and especially at high temperatures may be a driver or modulator of muscle-specific strategies where differential responses to the same stressor are observed, with preference given to muscles, such as the gastrocnemius, that have important post-aestivation locomotor functions (digging out of burrows, finding mates, avoiding predation). During aestivation, it is conceivable that 'excessive' degenerative atrophic pathways and cell death would be regulated against (van Breukelen et al., 2010). As a paucity of ROS also induces a state of cellular stress, it is not surprising that protective mechanisms did not completely abolish oxidative macromolecular modification in the muscles. It is thought that under physiological conditions the role of ROS may be overestimated (St-Pierre et al., 2002; Tahara et al., 2009), yet given the difficulties both in measuring ROS production (rather than emission) and in extrapolating this to the *in vivo* context (Andreyev et al., 2005), the importance of ROS to disuse atrophy is not always clear cut and is likely to vary with each case (Pellegrino et al., 2011). What is clear is that the mechanisms investigated here respond differently to temperature and differ greatly between jumping and non-jumping muscles. Thus, the present data suggest muscles of different function use variable biochemical regulation to avoid increased damage at higher temperatures during aestivation.

LIST OF ABBREVIATIONS

BSA	bovine serum albumin
HRP	horseradish peroxidase
HSP	heat shock protein
Hsp70	heat shock protein 70
MDA	muscle disuse atrophy
MDA-adduct	malondialdehyde-adduct
PBS	phosphate buffered saline
PMSF	phenylmethylsulfonyl fluoride
ROS	reactive oxygen species
SOD	superoxide dismutase
SSC	superoxide scavenging capacity
TAC	total antioxidant capacity

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