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

Karen M Young, Rebecca L Cramp, Craig E Franklin*

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

*Corresponding Author: c.franklin@uq.edu.au

Running Title: muscle-specific aestivation biochemistry

Keywords: disuse, muscle, antioxidant, Hsp70, ROS, oxidative damage, aestivation, temperature

ABSTRACT

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 rate of oxygen consumption likely increase the overall generation of ROS *in vivo*. Temperature-induced increases in muscle 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 muscles and small non-jumping muscles undergo atrophy seemingly commensurate with their rate of oxygen consumption during aestivation. However, since 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 the biochemical response to temperature would be muscle-specific. We examined the effect of temperature on the antioxidant and heat shock protein systems and evidence of oxidative damage to lipids and proteins in two functionally different skeletal muscles, gastrocnemius (jumping muscle) and iliofibularis (non-jumping muscle), by aestivating frogs at 24 and 30^{o}C for six months. We assayed small molecule antioxidant capacity, mitochondrial and cytosolic SOD and Hsp70 to show that protective mechanisms in disused muscles are differentially regulated both with respect to 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 of the different strategies muscles may use in regulating their oxidative environment during extended disuse and disuse at high temperature.

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 (Finkle and Holbrook, 2000; Powers et al., 2009) 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 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 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 (Constantini et al., 2010; Kavazis et al., 2009; Magaritis et al., 2009; Finkle 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 studies which show 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., 2009; Desaphy et al., 2010). 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 which 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 occurs.

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 six months of aestivation (dry season dormancy) at 30^{o}C significantly increases rate of oxygen consumption relative to frogs aestivating at 24^{o}C (Young et al., 2011). However, despite the increase in rate of oxygen consumption at 30^{o}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.

C. alboguttata aestivating for six months at either 24°C 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 heat shock proteins (Hsp70), and markers of oxidative damage (lipid peroxidation and protein carbonylation) in 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, (3) oxidative damage would occur during dormancy and to a greater degree in the iliofibularis.

MATERIALS AND METHODS

Experimental animals

Cyclorana alboguttata were collected from the districts of Dalby (S 27.18.169, E 151.26.206) and Theodore (S 24.94.743, E 150.07.529), 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 six 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 six 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 dissected out, gently blotted dry, and weighed. Muscles were placed in cryotubes and snap frozen in liquid nitrogen 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.

Total Antioxidant Capacity (TAC)

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 mM Tris HCL, 137 mM NaCl, 10% glycerol, 1% nonidet P-40 substitute, 2 mM EDTA) with protease

inhibitors added (Sigma P2714, PMSF, 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 (BIORAD Ultra Rocker, Hercules, CA, USA) then centrifuged (Beckman Coulter Allegra 25R, Brea, CA, USA) at 12000 rpm at 4°C for 20 min. The supernatant was aliquoted in 20 µl volumes and aliquots 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 ddH_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, Brea, CA, USA). Absorbance values were used to obtain nM Trolox equivalents from the standard curve and these were used to calculate the sample antioxidant capacity in nmol μl^{-1}. The TAC of each sample was expressed as nM mg^{-1} tissue.

Superoxide Dismutase (SOD)

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 mM HEPES, 1 mM EGTA, 210 mM mannitol, 70 mM sucrose, pH 7.2) with protease inhibitors added (Sigma P2714, PMSF) and homogenised with an Ika homogeniser (T10-Basic Ultra-Turrax®, IKA, Staufen, Germany). Tissue lysates were centrifuged (Beckman-Coulter Allegra 25R, Brea, CA, USA) at 4400 rpm for 5 min at 4°C. The supernatant was transferred to a fresh pre-weighed Eppendorf and centrifuged at 11300 rpm 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 superoxide dismutase 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 cytosolic samples required a 15% dilution to fall within the range of the standard curve, while mitochondrial samples were used undiluted. Ten µl 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 (Heidolph Unimax 1010, Schwabach, Germany) for 20 min at room temperature. Absorbance at 450nm was read using a microplate reader (Beckman Coulter DTX 880 Multimode Detector, Brea, CA, USA). The average absorbance from sample duplicates was used to calculate SOD activity in Units 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 Units 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 referred to in this manner throughout the results and discussion.

Heat-shock protein 70 (Hsp70)

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 1X extraction reagent (prepared from 5X reagent 80-1581, Enzo Life Sciences, Farmingdale, New York, USA) with added protease inhibitors (Sigma P2714, PMSF, Castle Hill, NSW, Australia) was added in a 1:2 (w/v, g/ml) ratio, followed by further grinding of tissues. Entire suspensions were transferred to fresh labelled polypropylene tubes and centrifuged (Beckman-Coulter Allegra 25R, Brea, CA, USA) at 21,000 g for 10 min at 4^{o}C. Supernatants containing the Hsp70 samples were retained in 10 µl aliquots and the remaining pellet was discarded. Aliquots were stored at -80^{o}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, Farmingdale, NY, USA) 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 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, Brea, CA, USA). Sample Hsp70 concentration was calculated from the equation obtained from the standard curve, accounting for the dilution factor (50). Results were 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 (137mM NaCl, 2.7mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4) with 5 µl ml^{-1} BHT (butylated hydroxytoluene) and protease inhibitors added (Sigma P2714, PMSF, Castle Hill, NSW, Australia), and homogenised with an Ika homogeniser (T10-Basic Ultra-Turrax®, IKA, Staufen, Germany). Tissue lysates were centrifuged (Beckman-Coulter Allegra 25R, Brea, California, USA) at 6000 rpm for 10 min at 4°C. The supernatant was aliquoted in 20 µl volumes and aliquots stored at -80°C until assay.

The levels of protein carbonyls were measured using a commercially available kit (STA-310, Cell Biolabs Inc., 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 concentration of samples were determined using a Qubit™ fluorometer (Invitrogen, Q32857, Mulgrave, VIC, Australia) and Quant-iT™ protein assay kit (Invitrogen, Q33211, Mulgrave, VIC, Australia) and samples were then

diluted in 0.1M PBS to 10 µg ml^{-1} protein. A standard curve was produced from 10 µg ml^{-1} oxidised 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 M PBS and then DNPH working solution was added to the wells and the plate 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 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 HRP conjugated secondary antibody was added to each well and incubated for 1 h at room temperature on an orbital shaker. Wells were washed 5 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, Brea, CA, USA). 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 MDA-adducts in skeletal muscle were measured using a commercially available kit (STA-332, Cell Biolabs Inc., San Diego, CA, USA) following the manufacturer's instructions. MDA-adducts are an advanced lipid peroxidation end product, formed from a natural by-product of lipid peroxidation, malondialdehyde (MDA), which when bound to protein form a stable adduct which can be used as a proxy for oxidative stress (Onorato et al., 1998; Requena et al., 1996). In brief, protein concentration of samples were determined using a Qubit™ fluorometer (Q32857, Invitrogen, Mulgrave, VIC, Australia) and Quant-iT™ protein assay kit (Q33211, Invitrogen, Mulgrave, VIC, Australia) 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} MDA-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 M PBS and then assay diluents were added to the wells and the plate left to incubate for 105 min at room temperature on an orbital shaker. Wells were washed three times with wash buffer and then anti-MDA antibody was added to the wells and incubated for1 h at room temperature on an orbital shaker. Wells were again washed and secondary antibody-HRP conjugate added to each well and incubation 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 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, Brea, CA, USA). The average absorbance of the sample duplicates was used to determine the amount of MDA-adducts. Results were expressed in pmol mg^{-1} protein.

Statistical Analyses

Data were assessed via a least squares means model considering metabolic state (2 levels: control and aestivator), temperature (2 levels: 24^{o}C and 30^{o}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 superoxide dismutase data. Within-treatment group, between-muscle comparisons were investigated using a least squares means model considering muscle type and the random variable of frog I.D., to account for the non-independence of comparing data from muscles obtained from the same source animals. Superoxide dismutase 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 Tukey-HSD. In the event that no differences were detected in the overall statistical model, planned pair-wise comparisons of data from frogs aestivating at 24^{o}C and 30^{o}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., 2011). The new standard errors were calculated by dividing the original errors by their associated means, squaring the result and summing those values for each variable. The square root was taken and then the value 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^{o}C relative to all other groups (Tukey-HSD, $P < 0.05$) and TAC from frogs aestivating at 30^{o}C was at the same level as that of the gastrocnemius of control frogs (Figure 1A). For the iliofibularis, TAC was significantly lower in muscles from aestivating frogs relative to control frogs ($F_{1,23}$ = 18.879, P = 0.0002) (Figure 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^{o}C aestivator group where the TAC was significantly higher in gastrocnemius muscle than in iliofibularis muscle ($F_{1,6}$ = 24.3782, P = 0.0026).

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

Superoxide Scavenging Capacity

For the gastrocnemius, there was a significant interaction between metabolic state and temperature on cytosolic superoxide scavenging capacity ($F_{1,24}$ = 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 superoxide scavenging capacity was significantly higher in frogs aestivating at 30^{o}C compared to those aestivating at 24^{o}C ($F_{1,12}$ = 5.8228, P = 0.0327)(Figure 3A). Mitochondrial superoxide scavenging capacity in the gastrocnemius was significantly higher in aestivating frogs than control frogs ($F_{1,24}$ = 15.8068, P = 0.0006) (Figure 3C). Comparing between aestivating groups, gastrocnemius

mitochondrial superoxide scavenging capacity was significantly higher in frogs aestivating at 30°C compared to those aestivating at 24°C ($F_{1,12}$ = 5.5878, P = 0.0358) (Figure 3C).

For the iliofibularis, cytosolic superoxide scavenging capacity was higher in aestivating frogs compared to controls ($F_{1,24}$ = 20.877, P = 0.0001) and also higher in both aestivating and control frogs at 30°C compared to 24°C ($F_{1,24}$ = 9.6521, P = 0.0048) (Figure 3B). Mitochondrial superoxide scavenging capacity was higher in iliofibularis from aestivating frogs compared to that of control frogs irrespective of temperature ($F_{1,22}$ = 9.0735, P = 0.0064) (Figure 3D).

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

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

Heat-shock protein70

For the gastrocnemius, Hsp70 levels were higher from aestivating frogs than control frogs ($F_{1,23}$ = 5.4635, P = 0.0285) (Figure 5A). In contrast, for the iliofibularis, Hsp70 levels were significantly lower in the iliofibularis from aestivating frogs compared to control frogs ($F_{1,24}$ = 40.9058, P <0.0001) (Figure 5B). Within the aestivating groups, iliofibularis Hsp70 levels were significantly lower in frogs aestivating at 30°C compared to frogs aestivating at 24°C ($F_{1,12}$ = 4.9130, P = 0.0467). Due to the small magnitude of the changes (3-5%), effect sizes

were calculated according to Coe (2002). For changes in response to aestivation, effect sizes for gastrocnemius at 24°C 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 (Figure 6A). For the iliofibularis, protein carbonyl levels were significantly higher in iliofibularis from aestivating frogs compared to control frogs ($F_{1,23} = 35.9678$, $P < 0.0001$) (Figure 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 of 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 (Figure 7A). In contrast, for the iliofibularis, lipid peroxidation was significantly lower in iliofibularis from aestivating frogs relative to control frogs ($F_{1,21} = 8.0293$, $P = 0.0099$) (Figure 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. Not only were significant differences apparent in some protective/reparative systems

in response to higher temperature, but 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 to 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 temperature effect on iliofibularis rate of oxygen consumption (Young et al., 2011).

The up-regulation 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 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 to 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, or (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 up-regulation 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 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 since other antioxidants, not measured here, will also be regulated. The changes in specifically mitochondrial SOD/SSC shown in this study however, may in part account for the lack of enhanced atrophy at 30^{o}C, since 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 approximately 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 to controls, irrespective of up or down-regulation 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 to 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 up-regulation 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 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 aestivator 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 up-regulating SOD/SSC in aestivation but up-regulating 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 suggest 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 shows that inducible Hsp70 was constitutively expressed in *C. alboguttata* gastrocnemius and iliofibularis muscle. The changes in Hsp70 with aestivation and, in the case of 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 down-regulated 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. Since *C. alboguttata* Hsp70 was regulated more so in response to aestivation than 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 compared to controls; consistent with hind-limb suspended mice where Hsp70 and other HSPs were down-regulated in the oxidative soleus muscle (Brocca et al., 2009).

Unlike 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* (2-3-fold) (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 iliofibularis of *C. alboguttata* experiences 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 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* one month of hypoxia resulted in a significant increase in Hsp70 in heart muscle but Hsp70 levels return to control values within four 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 a case were true for *C. alboguttata* a 'peak' in Hsp70 regulation may have been missed since measurements were only taken after six 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 enough 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 its role 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., 2009; 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.

Constitutive expression of Hsp70 possibly protects against expected accumulation of oxidative stress over prolonged aestivation, against the oxidative insult upon arousal, and presumably further assists *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 and 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^{o}C, though this assumes 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^{o}C and 30^{o}C despite an elevated rate of muscle oxygen consumption during aestivation at 30^{o}C (Young et al., 2011). However, unlike in the iliofibularis, the (measured) antioxidants were higher at 30^{o}C and possibly acted to reduce the oxidative insult sufficiently at 30^{o}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^{o}C or increased at 30^{o}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., 2009)

Since 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 since 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 and therefore might suggest 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 say 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 iliofibularis during aestivation. In the iliofibularis this may be attributed to the insensitivity of aestivating rate of oxygen consumption to temperature (Young et al., 2011) and therefore no 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 iliofibularis muscle, where carbonylation significantly increases. Increased carbonylation occurred despite the iliofibularis having greater antioxidants (those measured here) relative to presumed oxidative

insult from oxygen consumption during aestivation compared to 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^{o}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* 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 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 Nystrom, 1999; Oikawaa 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 down-regulation 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^{o}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)(Wood

and Storey, 2006). The highest protein carbonyl content in *C. alboguttata* was the significant increase in carbonyls in iliofibularis of aestivators at ~0.6 nmol mg^{-1} protein, while 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 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 iliofibularis relative to 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 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 burrow, 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). Since 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 over-estimated (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 suggests muscles of different function use variable biochemical regulation to avoid increased damage at higher temperatures during aestivation.

ACKNOWLEDGMENTS

KMY was supported by a University of Queensland Post-graduate scholarship. The authors wish to thank Emile McPhee for the care and maintenance of the frogs and three anonymous reviewers for comments on an earlier version of the manuscript. All experiments were conducted with the approval of the University of Queensland Animal Welfare Unit and the Queensland Environment Protection Agency.

FUNDING

Funding for this study was provided by the Australian Research Council (DP0666256 and DP110102976).

REFERENCES

Abele, D., Heise, K., Portner, H. O. and Puntarulo, S. (2002). Temperature-dependence of mitochondrial function and production of reactive oxygen species in the intertidal mud clam *Mya arenaria*. *J Exp Biol* **205**, 1831-1841.

Abravaya, K., Phillips, B. and Morimoto, R. (1991). Attenuation of the heat shock response in HeLa cells is mediated by the release of bound heat shock transcription factor and is modulated by changes in growth and in heat shock temperatures. *Genes Dev* **5**, 2117-2127.

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

Anderson, E. and Neufer, P. (2006). Type II skeletal myofibers possess unique properties that potentiate mitochondrial H_2O_2 generation. *Am J Physiol Cell Physiol* **290**, C844–C851.

Andreyev, A., Kushnareva, Y. and Starkov, A. (2005). Mitochondrial metabolism of reactive oxygen species. *Biochemsitry (Moscow)* **70**, 246-264.

Aruoma, O. (1998). Free Radicals, Oxidative Stress, and Antioxidants in Human Health and Disease. *J Am Oil Chem Soc* **75**, 199-212.

Balcerczyk, A. and Bartosz, G. (2003). Thiols are Main Determinants of Total Antioxidant Capacity of Cellular Homogenates. *Free Radical Research* **37**, 537–541.

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

Benov, L. (2001). How superoxide radical damages the cell. *Protoplasma* **217**, 33-36.

Bishop, T. and Brand, M. (2000). Processes contributing to metabolic depression in hepatopancreas cells from the snail *Helix aspersa*. *J Exp Biol* **203**, 3603–3612.

Brocca, L., Pellegriono, M., Desaphy, J.-F., Pierno, S., Camerino, D. and Bottinelli, R. (2009). Is oxidative stress a cause or consequence of disuse muscle atrophy in mice? A proteomic approach in hindlimb-unloaded mice. *Exp Physiol* **95**, 331-350.

Cadenas, E. and Davies, K. (2000). Mitochondrial free radical generation, oxidative stress, and aging. *Free Rad Biol Med* **29**, 222-230.

Carey, H., Sills, N. and Gorham, D. (1999). Stress proteins in mammalian hibernation. *Am Zool* **39**, 825-835.

Chapovetsky, V. and Katz, U. (2006). Hsp levels in toad heart are independent of temperature and pharmacological stimuli. *Ital J Zool* **73**, 309-317.

Coe, R. (2002). It's the effect size, stupid- What effect size is and why its important. In *Annual Conference of the British Educational Research Association*. England: University of Exeter.

Constantini, D., Rowe, M., Butler, M. and McGraw, K. (2010). From molecules to living systems: historical and contemporary issues in oxidative stress and antioxidant ecology. *Funct Ecol* **24**, 950–959.

Cooper, R. (1972). Alterations during immobilization and regeneration of skeletal muscle in cats. *J Bone Joint Surg* **54,** 919–953.

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

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.

Currie, S. and Boutilier, R. (2001). Strategies of hypoxia and anoxia tolerance in cardiomyocytes from the overwintering common frog, *Rana temporaria*. *Physiol Biochem Zool* **74**, 420–428.

Dalle-Donne, I., Rossi, R., Giustarini, D., Gagliano, N., Lusini, L., Milzani, A., Di Simplicio, P. and Colombo, R. (2001). Actin carbonylation: From a simple marker of protein oxidation to relevant signs of functional impairment *Free Radical Biology and Medicine* **31**, 1075–1083.

Dalle-Donne, I., Rossi, R., Giustarini, D., Milzani, A. and Colombo, R. (2003). Protein carbonyl groups as biomarkers of oxidative stress. *Clin Chim Acta* **329**, 23-38.

Desaphy, J.-F., Pierno, S., Liantonio, A., Giannuzzi, V., Digennaro, C., Dinardo, M., Camerino, G., Ricciuti, P., Brocca, L., Pellegrino, M. et al. (2010). Antioxidant treatment of hindlimb-unloaded mouse counteracts fiber type transition but not atrophy of disused muscles. *Pharmacol Res* **61**, 553-563.

Desplanches, D., Echochard, L., Sempore, B., Mayet-Sornay, M.-H. and Favier, R. (2004). Skeletal muscle HSP72 response to mechanical unloading: influence of endurance training. *Acta Physiol Scand* **180**, 387-394.

Dukan, S. and Nystrom, T. (1999). Oxidative Stress Defense and deterioration of growth-arrested *Escherichia coli* cells. *J Biol Chem* **274**, 26027–26032.

Feder, M. E. and Hofmann, G. (1999). Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. *Ann Rev Physiol* **61**, 243-82.

Ferreira-Cravo, M., Welker, A. and Hermes-Lima, M. (2010). Chapter 3 The Connection Between Oxidative Stress and Estivation in Gastropods and Anurans. In *Aestivation: Molecular and Physiological Aspects*, eds. C. Navas and J. Carvalho). Berlin, Heidelberg: Springer-Verlag.

Ferreira, M. V. R., Alencastro, A. C. R. and Hermes-Lima, M. (2003). Role of antioxidant defences during estivation and anoxia exposure in the freshwater snail Biomphalaria tenagophila (Orbigny, 1835). *Canadian Journal of Zoology* **81**, 1239.

Finkle, T. and Holbrook, N. (2000). Oxidants, oxidative stress and the biology of ageing. *Nature* **408**, 239-247.

Flanagan, S., Ryan, A., Gisolfi, C. and Moseley, P. (1995). Tissue-specific HSP70 response in animals undergoing heat stress. *Am J Physiol Reg Integ Comp Physiol* **37**, R28-R33.

Folkesson, M., Mackey, A., Holm, L., Kjaer, M., Paulsen, G., Raastad, T., Henriksson, J. and Kadi, F. (2008). Immunohistochemical changes in the expression of HSP27 in exercised human vastus lateralis muscle. *Acta Physiologica* **194**, 215-222.

Fredriksson, A., Ballesteros, M., Dukan, S. and Nyström, T. (2005). Defense against protein carbonylation by DnaK/DnaJ and proteases of the heat shock regulation. *J Bacteriol* **187**, 4207-4213

Fulle, S., Protasi, F., Di Tano, G., Pietrangelo, T., Beltramin, A., Boncompagni, S., Vecchiet, L. and Fano, G. (2004). The contribution of reactive oxygen species to sarcopenia and muscle ageing. *Exp Gerontol* **39**, 17-24.

Grably, S. and Piery, Y. (1981). Weight and tissue changes in longterm starved frogs *Rana esculenta Comp Biochem Physiol* **69A**, 683-688.

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

Halliwell, B. and Whiteman, M. (2004). Measuring reactive species and oxidative damage in vivo and in cell culture: how should you do it and what do the results mean? *Brit J Pharmacol* **142**, 231-255.

Hamanaka, R. and Chandel, N. (2010). Mitochondrial reactive oxygen species regulate cellular signalling and dictate biological outcomes. *Trends Biochem Sci* **35**, 505–513.

Han, D., Antunes, F., Canali, R., Rettori, D. and Cadenas, E. (2003). Voltage-dependent anion channels control the release of the superoxide anion from mitochondria to cytosol. *J Biol Chem* **278**, 5557-5563.

Heise, K., Puntarulo, S., Portner, H. O. and Abele, D. (2003). Production of reactive oxygen species by isolated mitochondria of the Antarctic bivalve *Laternula elliptica* (King and Broderip) under heat stress. *Comp Biochem Physiol C* **134**, 79-90.

Hermes-Lima, M. and Storey, K. (1995). Antioxidant defences and metabolic depression in a pulmonate land snail. *Am J Physiol Reg Integ Comp Physiol* **268**, 1386-1393.

Hoppe, G., Chai, Y., Crabb, J. and Sears, J. (2004). Protein s-glutathionylation in retinal pigment epithelium converts heat shock protein 70 to an active chaperone. *Exp Eye Res* **78**, 1085–1092.

Hudson, N. J. and Franklin, C. E. (2002). Maintaining muscle mass during extended disuse: Aestivating frogs as a model species. *Journal of Experimental Biology* **205**, 2297-2303.

Jastroch, M., Divakaruni, A., Mookerjee, S., Treber, J. and Brand, M. (2010). Mitochondrial proton and electron leaks. *Essays Biochem* **47**, 53-67.

Jones, D. (2008). Radical-free biology of oxidative stress. *Am J Physiol Cell Physiol* **295**, C849-C868.

Kalmar, B. and Greensmith, L. (2009). Induction of heat shock proteins for protection against oxidative stress. *Adv Drug Del Rev* **61**, 310-318.

Kamata, H. and Hirata, H. (1999). Redox regulation of cellular signalling. *Cell Signal* **11**, 1-14.

Kavazis, A., Talbert, E., Smuder, A., Hudson, M., Nelson, W. and Powers, S. (2009). Mechanical ventilation induces diaphragmatic mitochondrial dysfunction and increased oxidant production. *Free Rad Biol Med* **46**, 842-450.

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

Kondo, H., Miura, M. and Itokawa, Y. (1991). Oxidative stress in skeletal muscle atrophied by immobilisation. *Acta Physiol Scand* **142**, 527-528.

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

Krivoruchko, A. and Storey, K. (2010). Mechanisms of natural anoxia tolerance and potential links to longevity. *Oxid Med Cell Longev* **3**, 186-198.

L'Vova, S. P. and Gasangadzhieva, A. G. (2003). Lipid peroxidation and status of the antioxidant system in tissues of little suslik (*Citellus pygmaeus*) in the course of hibernation. *Biol Bull* **30**, 547-550.

Lawler, J. M., Song, W. and Demaree, S. R. (2003). Hindlimb unloading increases oxidative stress and disrupts antioxidant capacity in skeletal muscle. *Free Radical Biology and Medicine* **35**, 9-16.

Lee, K., Park, J. Y., Yoo, W., Gwag, T., Lee, J.-W., Byun, M.-W. and Choi, I. (2008). Overcoming muscle atrophy in a hibernating mammal despite prolonged disuse in dormancy: Proteomic and molecular assessment. *J Cell Biochem* **104**, 642 - 656.

Liu, Y., Fiskum, G. and Schubert, D. (2002). Generation of reactive oxygen species by the mitochondrial electron transport chain. *J Neurochem* **80**, 780-787.

Liu, Y. and Steinacker, J. (2001). Changes in skeletal muscle heat shock proteins: pathological significance. *Front Biosci* **6**, 12-25.

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.

Locke, M., Noble, E. and Atkinson, B. (1991). Inducible isoform of HSP70 is constitutively expressed in a muscle fiber type specific pattern. *Am J Phyisol* **261**, C774-779.

Magaritis, I., Rousseau, A., Marini, J. and Chopard, A. (2009). Does antioxidant system adaptive response alleviate related oxidative damage with long term bed rest? *Clin Biochem* **42**, 371–379.

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 B* **180**, 1033-1043.

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

Martin, K. and Barrett, J. (2002). Reactive oxygen species as double-edged swords in cellular processes: low-dose cell signalling versus high-dose toxicity. *Human Exp Toxicol* **21**, 71-75.

Masuda, K., Tanabe, K., Kuno, S., Hirayama, A. and Nagase, S. (2003). Antioxidant capacity in rat skeletal muscle tissues determined by electron spin resonance. *Comp Biochem Physiol B* **134**, 215-220.

McClung, J., Judge, A., Talbert, E. and Powers, S. (2009). Calpain-1 is required for hydrogen peroxide-induced myotube atrophy. *American Journal of Physiology - Cell Physiology* **296**, C363–C371.

McDonagh, J. C., Callister, R. J., Favron, M. L. and Stuart, D. G. (2004). Resistance to disuse atrophy in a turtle hindlimb muscle. *J Comp Physiol C* **190**, 321-329.

Mecocci, P., Fan´o, G., Fulle, S., Macgarvey, U., Shinobu, L., Polidori, M., Cherubini, A., Vecchiet, H., Senin, U. and Beal, M. (1999). Age-dependent increases in oxidative damage to DNA, lipids, and proteins in human skeletal muscle. *Free Rad Biol Med* **26**, 303-308.

Min, K., Smuder, A., Kwon, O., Kavazis, A., Szeto, H. and Powers, S. (2011). Mitochondrial-targeted antioxidants protect the skeletal muscle against immobilization-induced muscle atrophy. *J Appl Physiol* **111**, 1459-1466

Muchowski, P. and Wacker, J. (2005). Modulation of neurodegeneration by molecular chaperones. *Nature Rev Neurosci* **6**, 11-22.

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**, R1159-1168.

Murphy, M. (2009). How mitochondria produce reactive oxygen species. *Biochem J* **417**, 1-13.

Murphy, M., Holmgren, A., Larsson, N., Halliwell, B., Chang, C., Kalyanaraman, B., Goo Rhee, S., Thornalley, P., Partridge, L., Gems, D. et al. (2011). Unravelling the biological roles of reactive oxygen species. *Cell Metabol* **13**, 361-366.

Nyström, T. (2005). Role of oxidative carbonylation in protein quality control and senescence. *EMBO J* **24**, 1311-1317.

Oikawaa, S., Yamadaa, T., Minohatab, T., Kobayashia, H., Furukawad, A., Tada-Oikawaa, S., Hirakua, Y., Murataa, M., Kikuchie, M. and Yamashimaf, T. (2009). Proteomic identification of carbonylated proteins in the monkey hippocampus after ischemia–reperfusion. *Free Rad Biol Med* **46**, 1472-1477

Onorato, J., Thorpe, S. and Baynes, J. (1998). Immunohistochemical and ELISA Assays for Biomarkers of Oxidative Stress in Aging and Disease. *An NY Acad Sci* **854**, 277-290.

Ott, M., Gogvadze, V., Orrenius, S. and Zhivotovsky, B. (2007). Mitochondria, oxidative stress and cell death. *Apoptosis* **12**, 913-922.

Pellegrino, M., Desaphy, J.-F., Brocca, L., Pierno, S., Camerino, D. and Bottinelli, R. (2011). Redox homeostasis, oxidative stress and disuse muscle atrophy. *J Physiol* **589**, 2147-2160.

Powers, S., Duarte, J., Kavazis, A. and Talbert, E. (2009). Reactive oxygen species are signalling molecules for skeletal muscle adaptation. *Exp Physiol* **95**, 1-9.

Powers, S., Wiggs, M., Duarte, J., Zergeroglu, A. and Demirel, H. (2012). Mitochondrial signalling contributes to disuse muscle atrophy. *Am J Physiol*. **doi:10.1152/ajpendo.00609.2011**.

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

Requena, J., Fu, M., Ahmed, M., Jenkins, A., Lyons, T. and Thorpe, S. (1996). Lipoxidation products as biomarkers of oxidative damage to proteins during lipid peroxidation reactions. *Nephrol Dialysis Transplant* **11**, 48-53.

Richter, C., Park, J. and Ames, B. (1988). Normal oxidative damage to mitochondrial and nuclear DNA is extensive. *Proc Nat Acad Sci USA* **85**, 6465-6467.

Sarsour, E., Venkataraman, S., Kalen, A., Oberley, L. and Goswami, P. (2008). Manganese superoxide dismutase activity regulates transitions between quiescent and proliferative growth. *Aging Cell* **7**, 405–417.

Selsby, J. T. and Dodd, S. L. (2005). Heat treatment reduces oxidative stress and protects muscle mass during immobilization. *Am J Physiol* **289**, R134-R139.

Seo, Y., Lee, K., Park, K., Bae, K. and Choi, I. (2006). A Proteomic Assessment of Muscle Contractile Alterations during Unloading and Reloading. *J Biochem* **139,** 71–80.

Servais, S., Letexier, D., Favier, R., Duchamp, C. and Desplanches, D. (2007). Prevention of unloading-induced atrophy by vitamin E supplementation: Links between oxidative stress and soleus muscle proteolysis? *Free Rad Biol Med* **42**, 627-635.

Sharp, P., Krishnan, M., Pullar, O., Navarrete, R., Wells, D. and de Belleroche, J. (2006). Heat shock protein 27 rescues motor neurons following nerve injury and preserves muscle function. *Exp Neurol* **198**, 511-518.

Shavlakadze, T. and Grounds, M. (2006). Of bears, frogs, meat, mice and men: complexity of factors affecting skeletal muscle mass and fat. *BioEssays* **28**, 994-1009.

Sørensen, J., Kristensen, T. and Loeschcke, V. (2003). The evolutionary and ecological role of heat shock proteins. *Ecol Lett* **6**, 1025–1037.

St-Pierre, J., Buckingham, J., Roebuck, S. and Brand, M. (2002). Topology of superoxide production from different sites in the mitochondrial electron transport chain. *J Biol Chem* **277**, 44784–44790.

Storey, K. (1996). Oxidative stress: animal adaptations in nature *Braz J Med Biol Res* **29**, 1715-1733.

Storey, K. and Storey, J. (2011). Heat shock proteins and hypometabolism: adaptive strategy for proteome preservation. *Res Reports Biol* **2011**, 57-68.

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.

Tahara, E., Navarete, F. and Kowaltowski, A. (2009). Tissue-, substrate-, and site-specific characteristics of mitochondrial reactive oxygen species generation. *Free Rad Biol Med* **46**, 1283-1297.

Tamarit, J., Cabiscol, E. and Ros, J. (1998). Identification of the major oxidatively damaged proteins in *Escherichia coli* cells exposed to oxidative stress. *J Biol Chem* **273**, 3027–3032.

Turrens, J. F. (2003). Mitochondrial formation of reactive oxygen species. *J Physiol* **552**, 335-344.

Valentine, J. S., Wertz, D. L., Lyons, T. J., Liou, L. L., Goto, J. J. and Gralla, E. B. (1998). The dark side of dioxygen biochemistry. *Curr Opin Chem Biol* **2**, 253-262.

van Breukelen, F., Krumschnabel, G. and Podrabsky, J. (2010). Vertebrate cell death in energy-limited conditions and how to avoid it: what we might learn from mammalian hibernators and other stress-tolerant vertebrates. *Apoptosis* **15**, 386-399.

Wallen, E., Buettner, G. and Moseley, P. (1997). Oxidants differentially regulate heat shock response. *International Journal of Hyperthermia* **13**, 517-524.

Weydert, C. and Cullen, J. (2010). Measurement of superoxide dismutase, catalase and glutathione peroxidase in cultured cells and tissue. *Nature Protocols* **5**, 51-66.

Withers, P. and Guppy, M. (1996). Do Australian desert frogs co-accumulate counteracting solutes with urea during aestivation? *J Exp Biol* **199**, 1809–1816.

Wood, A. and Storey, K. (2006). Vertebrate freezing survival: Regulation of the multicatalytic proteinase complex and controls on protein degradation. *Biochim Biophys Acta* **1760**, 395–403.

Yan, L. and Sohal, R. S. (2002). Analysis of oxidative modification of proteins. In *Curr Protoc Cell Biol*, vol. supp 14.

Young, K. M., Cramp, R. L., White, C. R. and Franklin, C. E. (2011). Influence of elevated temperature on metabolism during aestivation: implications for muscle disuse atrophy. *J Exp Biol* **214**, 3782-3789.

Young, K.M., Cramp, R.L., Franklin, C.E. (2012). Hot and steady: elevated temperatures do not enhance muscle disuse atrophy during prolonged aestivation in the ectotherm *Cyclorana alboguttata*. *J Morph* doi: 10.1002/jmor.20080

Yu, Z., Magee, W. and Spotila, J. (1994). Monoclonal antibody ELISA test indicates that large amounts of constitutive Hsp-70 are present in salamanders, turtles and fish *J Therm Biol* **19**, 41-53.

Zhang, P., Chen, X. and Fan, M. (2007). Signalling mechanisms involved in disuse muscle atrophy. *Med Hypoth* **69**, 310-321.

FIGURE LEGENDS

Figure 1. Total Antioxidant Capacity (TAC) of A. gastrocnemius and B. iliofibularis muscles. Data are expressed as means ± s.e.m. Significant parameters from the analysis are inset on each graph (see text for models). Letters detail the results of *post hoc* analysis on a significant interaction term. Bars not connected by the same letter 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^{o}C aestivator group for both graphs which are N= 6.

Figure 2. Total Antioxidant Capacity (TAC) standardised to muscle rate of oxygen consumption of A. gastrocnemius and B. iliofibularis muscles. 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 Figure 1 and muscle oxygen consumption data from (Young et al., 2011) statistical significance cannot be indicated. See methods for calculation.

Figure 3. Superoxide scavenging capacity in A. gastrocnemius cytosolic fraction, B. iliofibularis cytosolic fraction, C. gastrocnemius mitochondrial fraction, and D. iliofibularis mitochondrial fraction. 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 least squares means model (within the cellular fraction). * indicates a significant difference between 30^{o}C aestivator and 24^{o}C aestivator values as determined by planned comparison ANOVA. In all graphs each bar is N= 7 except for graph D where the 24^{o}C and 30^{o}C aestivator bars are N= 6.

Figure 4. Superoxide scavenging capacity standardised to muscle rate of oxygen consumption for A. gastrocnemius cytosolic fraction, B. iliofibularis cytosolic fraction, C. gastrocnemius mitochondrial fraction, and D. iliofibularis mitochondrial fraction. 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 Figure 3 and muscle oxygen consumption data from (Young et al., 2011) statistical significance cannot be indicated. See methods for calculation.

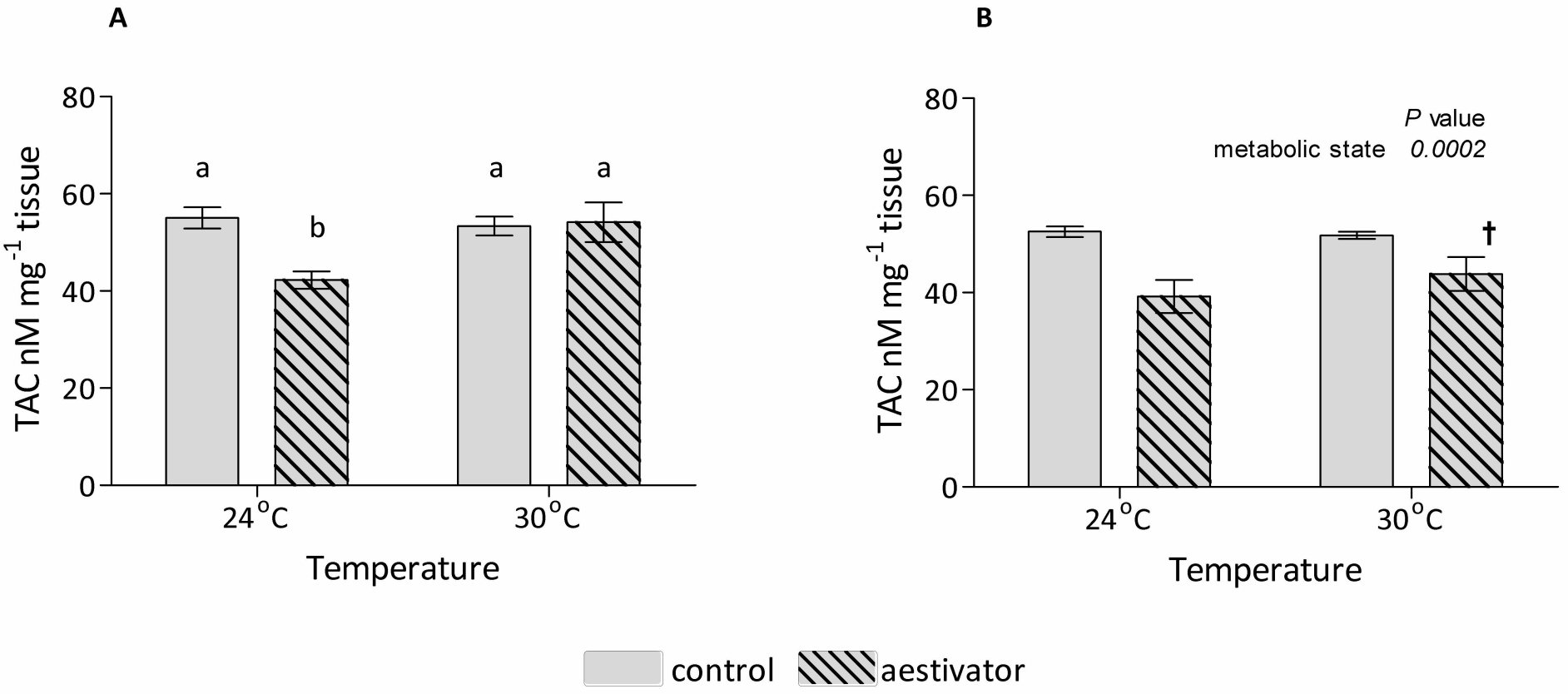
Figure 5. Heat-Shock Protein 70 (Hsp70) of A. gastrocnemius and B. iliofibularis muscles. Data are expressed as means ± s.e.m. Significant parameters from the analysis are inset on each graph (see text for models). † indicates a significant difference of the iliofibularis value from the gastrocnemius value for the associated treatment group as determined by least squares means model. * indicates a significant difference between 30^{o}C aestivator and 24^{o}C aestivator values as determined by planned comparison ANOVA. Note: y-axis scales are different in graphs A and B. Sample sizes for all bars are N= 7 except for the 24^{o}C control bar in graph A which is N = 6.

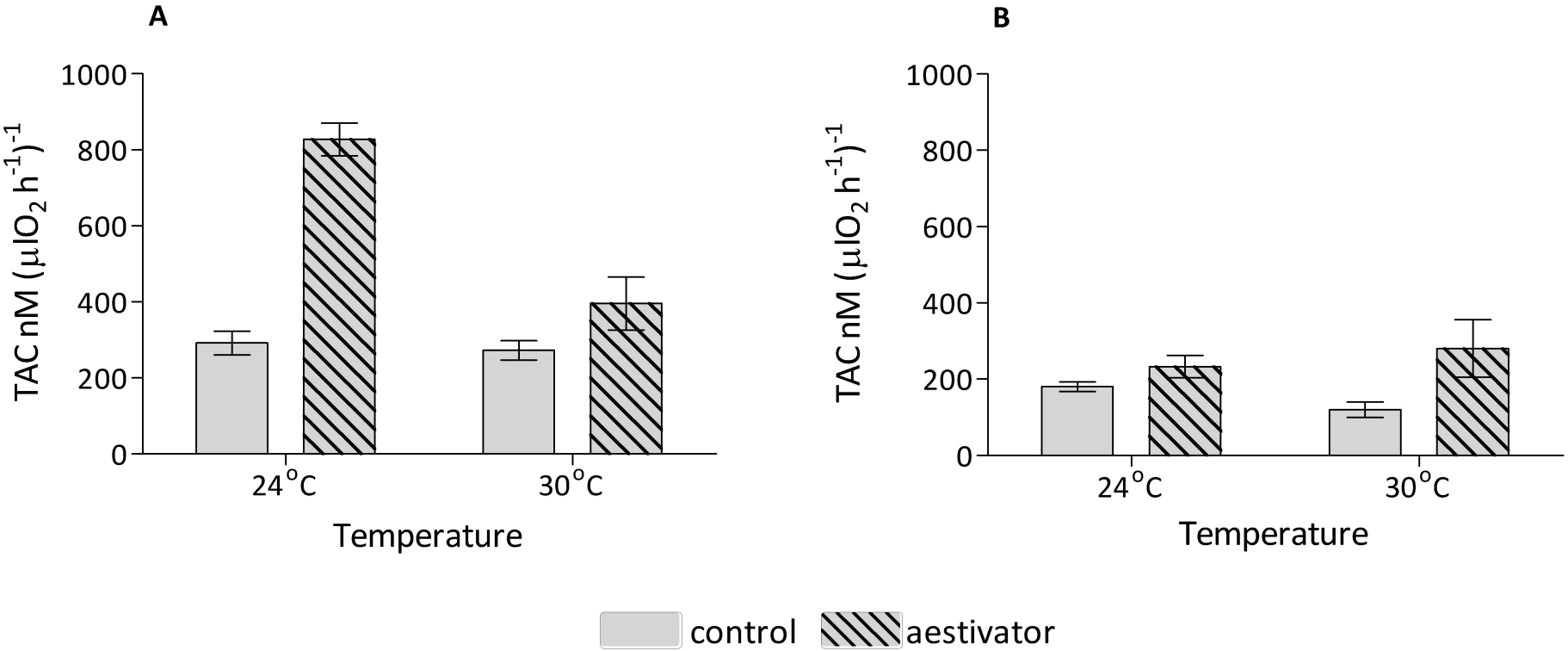
Figure 6. Protein Carbonyl content of A. gastrocnemius and B. iliofibularis muscles. Data are expressed as means ± s.e.m. Significant parameters from the analysis are inset on each graph (see text for models). † indicates a significant difference of the iliofibularis value from the gastrocnemius value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

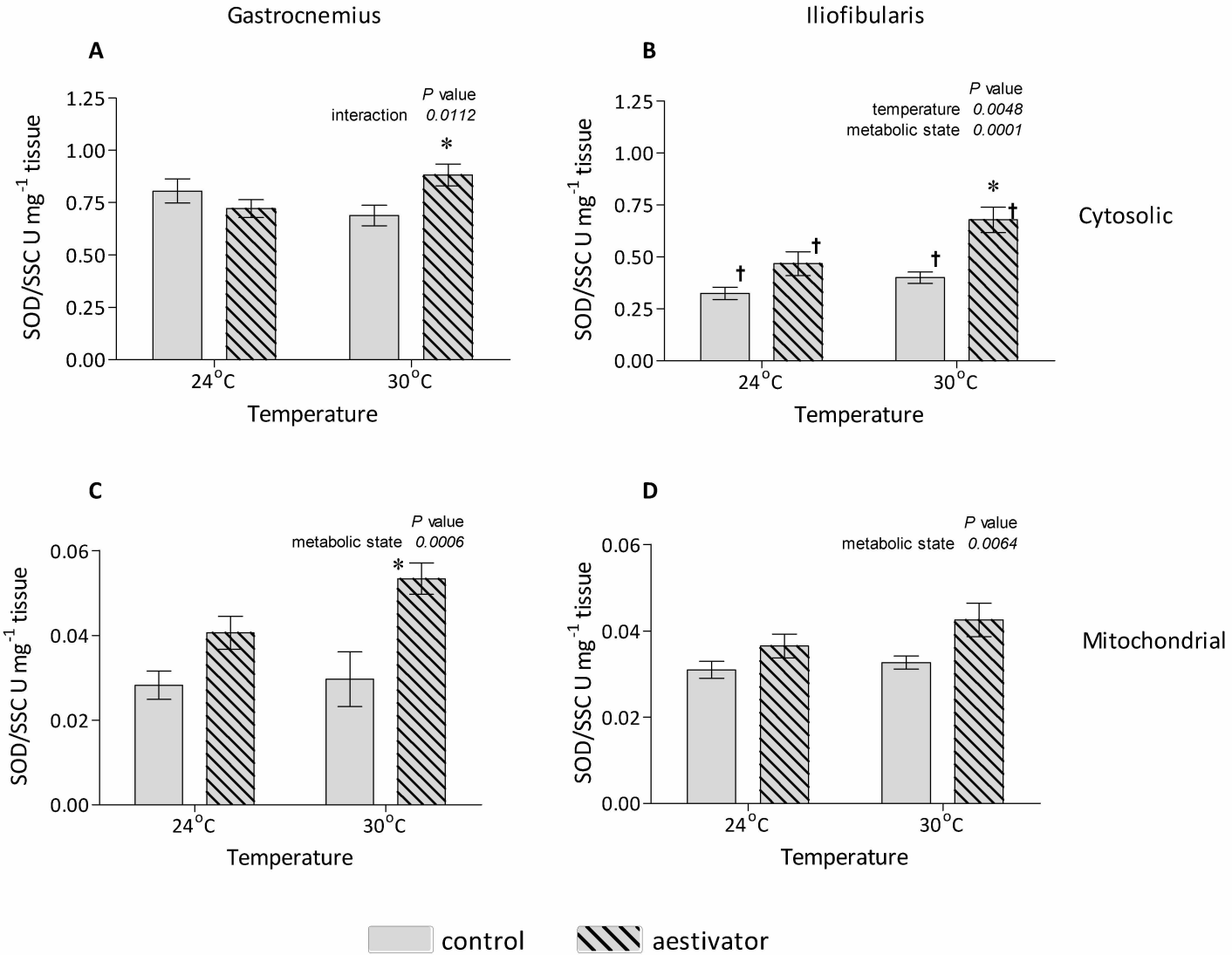
Figure 7. MDA-adduct content of A. gastrocnemius and B. iliofibularis muscles. Data are expressed as means ± s.e.m. Significant parameters from the analysis are inset on each graph (see text for models). † indicates a significant difference of the iliofibularis value from the gastrocnemius value for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

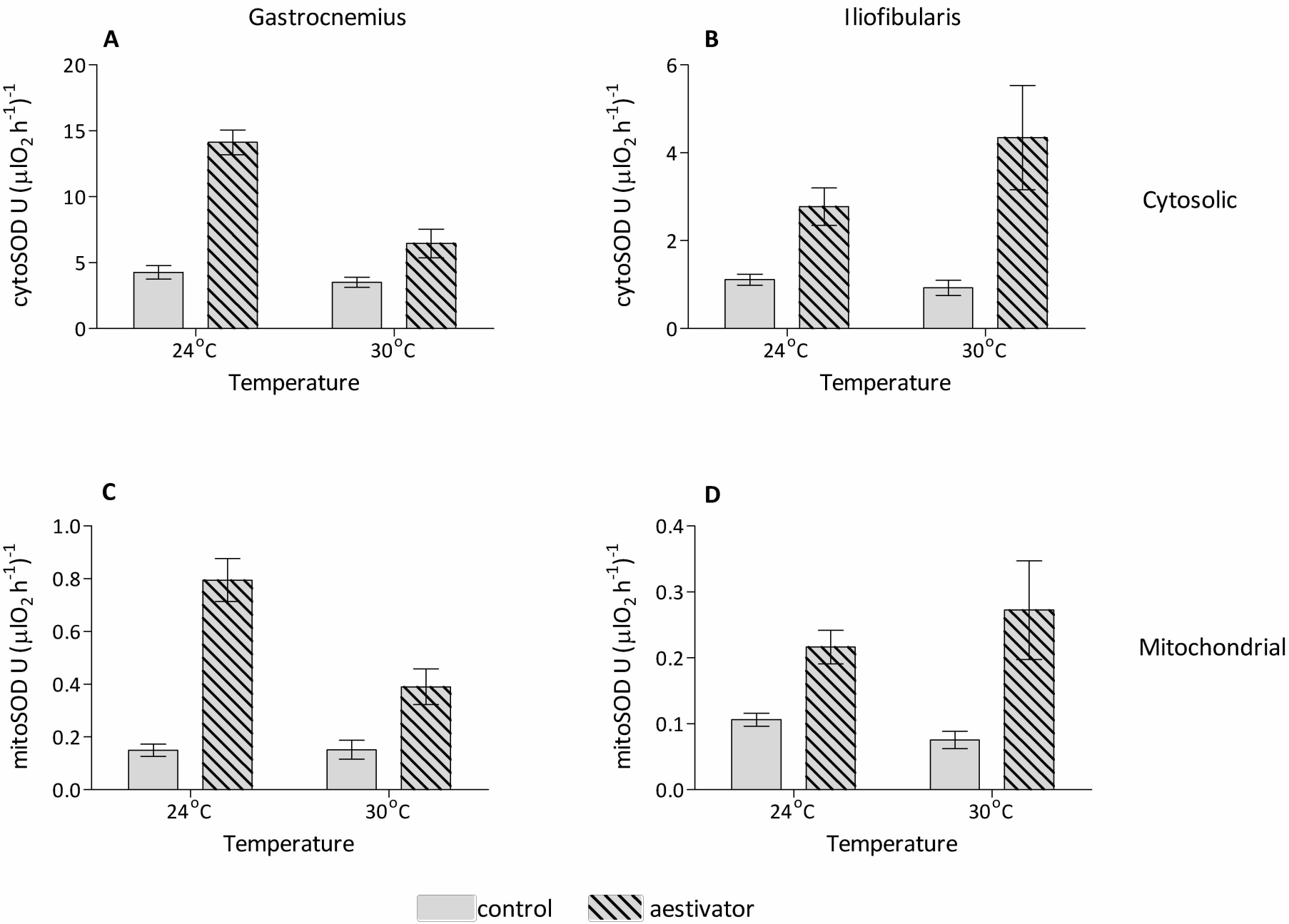
TABLE LEGENDS

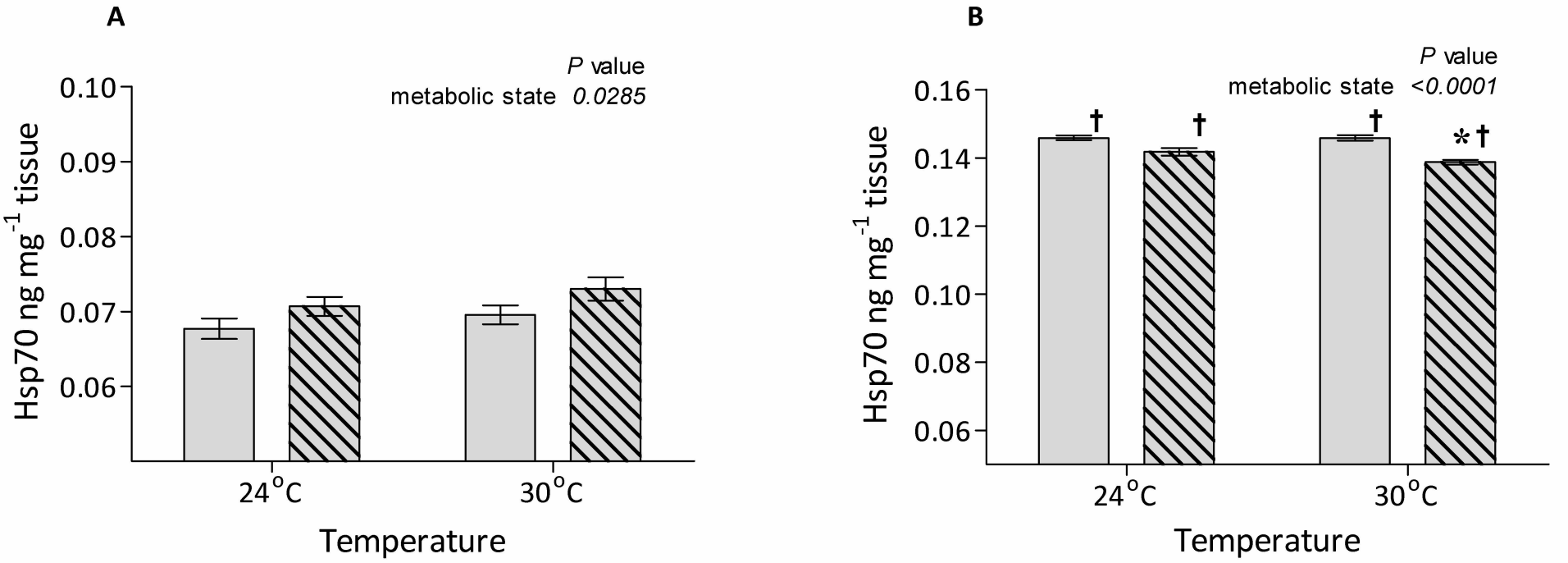
Table 1. Summary of the data. Results are divided into three main comparisons of interest. For comparisons (1) and (2) upwards arrows indicate an increase in the parameter, downwards arrows indicate a decrease in the parameter, horizontal lines indicate no change. For comparison (3) ‘I’ refers to iliofibularis,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

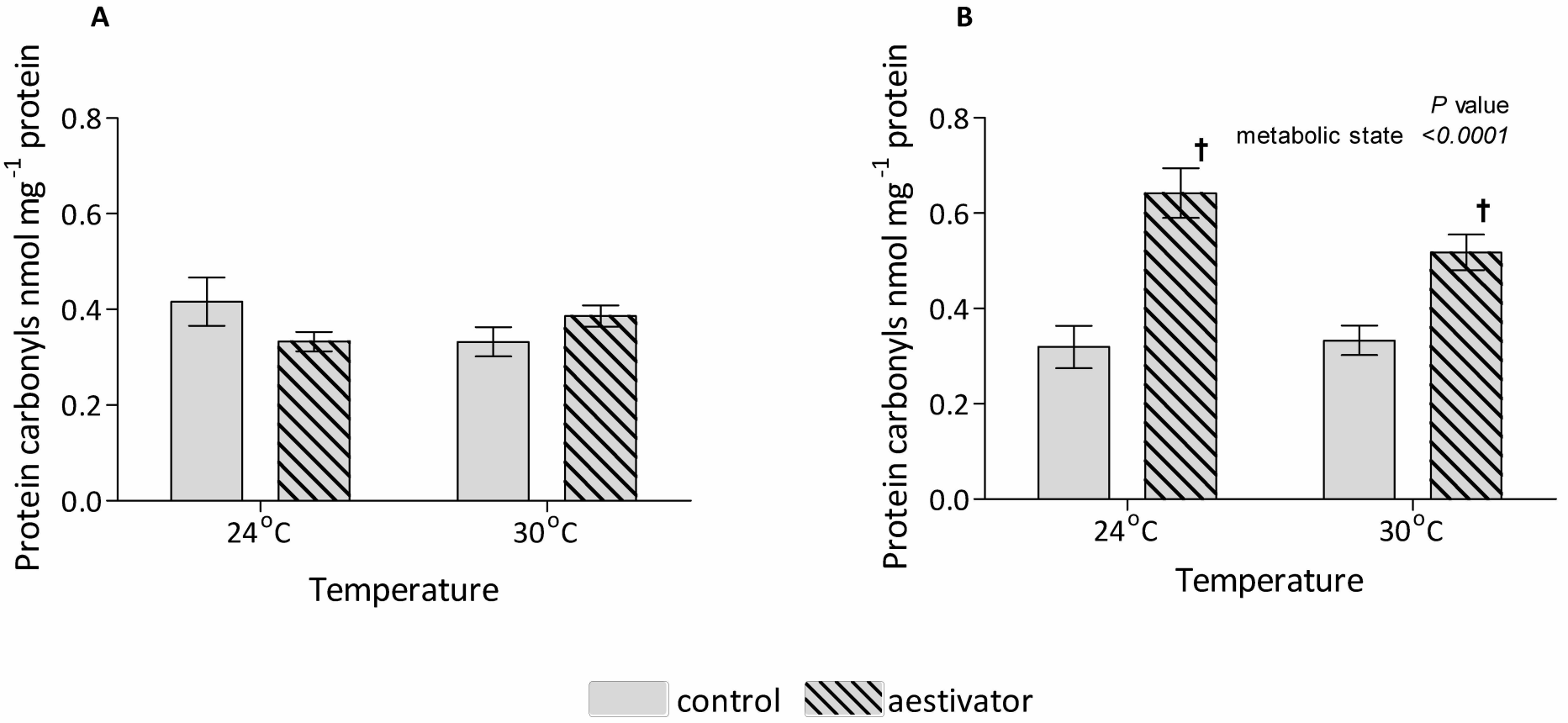

A
B
80
60
40
20
0
TAC nM mg-1 tissue
a
b
a
a
24°C
30°C
Temperature
P value
metabolic state 0.0002
†
control
aestivator

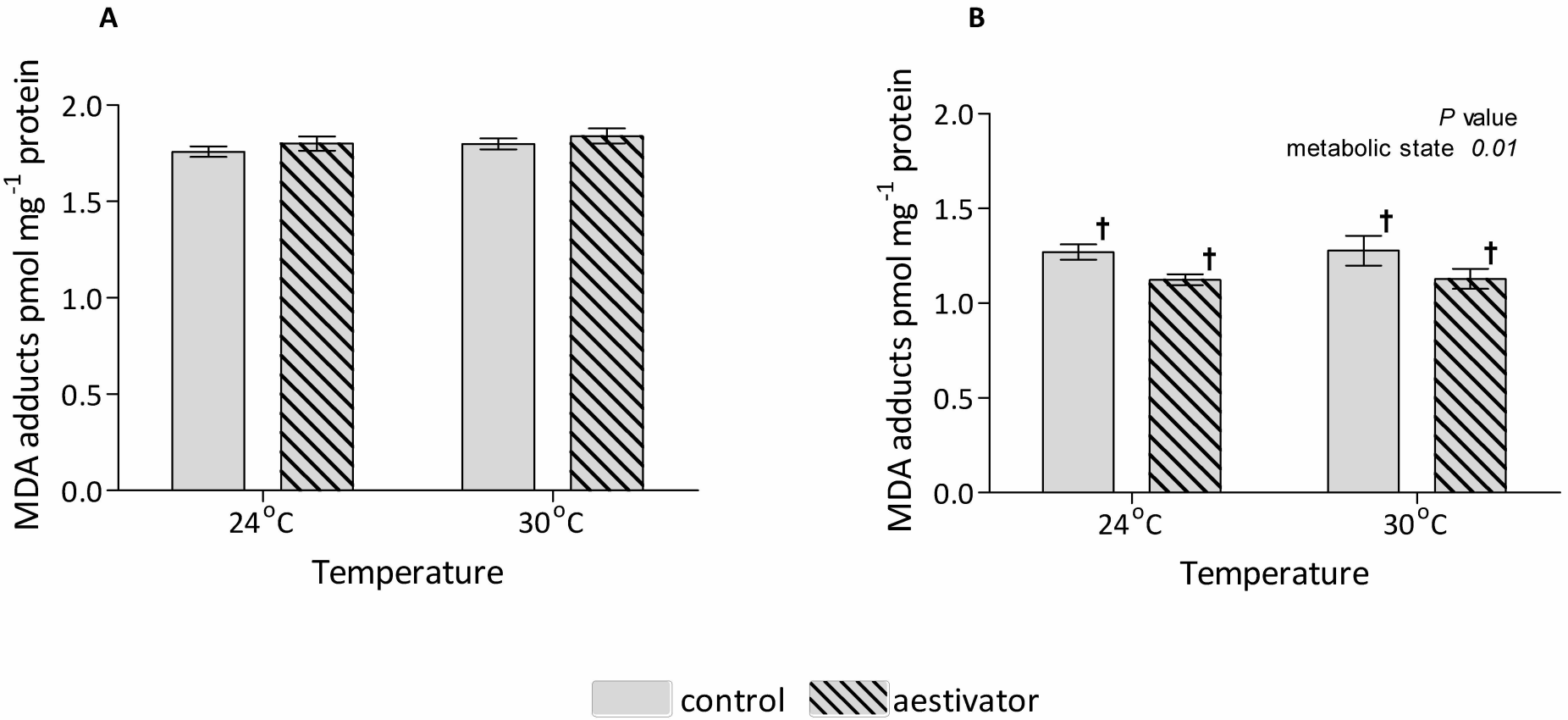

A
B
1000
800
600
400
200
0
TAC nM (µlO2 h-1)-1
24°C
30°C
Temperature
control
aestivator


Gastrocnemius
Iliofibularis
A
B
C
D
P value
interaction 0.0112
P value
temperature 0.0048
metabolic state 0.0001
P value
metabolic state 0.0006
P value
metabolic state 0.0064
SOD/SSC U mg-1 tissue
1.25
1.00
0.75
0.50
0.25
0.00
0.06
0.04
0.02
24°C
30°C
Temperature
Cytosolic
Mitochondrial
control
aestivator


Gastrocnemius
Iliofibularis
A
B
C
D
cytoSOD U (µlO2 h-1)-1
mitoSOD U (µlO2 h-1)-1
24°C
30°C
Temperature
Cytosolic
Mitochondrial
control
aestivator


A
P value
metabolic state 0.0285
0.10
0.09
0.08
0.07
0.06
Hsp70 ng mg-1 tissue
24°C
30°C
Temperature
B
P value
metabolic state <0.0001
0.16
0.14
0.12
0.10
0.08
0.06
Hsp70 ng mg-1 tissue
†
†
†
*†
24°C
30°C
Temperature
control
aestivator


A
B
Protein carbonyls nmol mg -1 protein
0.0
0.2
0.4
0.6
0.8
24°C
30°C
Temperature
P value
metabolic state <0.0001
†
†
control
aestivator


A
B
MDA adducts pmol mg-1 protein
2.0
1.5
1.0
0.5
0.0
24°C
30°C
Temperature
P value
metabolic state 0.01
†
control
aestivator

Comparison	Group/ Muscle	Protective Mechanisms				Oxidative Damage	
		Antioxidant capacity	Cytosolic SOD/SSC	Mitochondria SOD/SSC	Hsp70	Lipid oxidation	Protein oxidation
(1) *Within* aestivation, with *higher* temperature	Gastroc	↑	↑	↑	—	—	—
	Iliofib	—	↑	—	↓	—	—
(2) With transition from non-aestivating to aestivating		*(24°C)/(30°C)*					
	Gastroc	↓	—	↑	↑	—	—
	Iliofib	↓	↑	↑	↓	↓	↑
(3) Within aestivation and temperature	24°C Aest	=	G > I	=	I > G	G > I	I > G
	30°C Aest	G > I	G > I	=	I > G	G > I	I > G