The Journal of Experimental Biology 212, 3664-3672
Published by The Company of Biologists 2009
doi:10.1242/jeb.033688

Skeletal muscle atrophy occurs slowly and selectively during prolonged aestivation in *Cyclorana alboguttata* (Günther 1867)

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Accepted 19 August 2009

SUMMARY

We investigated the effect of prolonged immobilisation of six and nine months duration on the morphology and antioxidant biochemistry of skeletal muscles in the amphibian aestivator *Cyclorana alboguttata*. We hypothesised that, in the event of atrophy occurring during aestivation, larger jumping muscles were more likely to be preserved over smaller non-jumping muscles. Whole muscle mass (g), muscle cross-sectional area (CSA) (μm^2), water content (%) and myofibre number (per mm^2) remained unchanged in the cruralis muscle after six to nine months of aestivation; however, myofibre area (μm^2) was significantly reduced. Whole muscle mass, water content, myofibre number and myofibre CSA remained unchanged in the gastrocnemius muscle after six to nine months of aestivation. However, iliofibularis dry muscle mass, whole muscle CSA and myofibre CSA was significantly reduced during aestivation. Similarly, sartorius dry muscle mass, water content and whole muscle CSA was significantly reduced during aestivation. Endogenous antioxidants were maintained at control levels throughout aestivation in all four muscles. The results suggest changes to muscle morphology during aestivation may occur when lipid reserves have been depleted and protein becomes the primary fuel substrate for preserving basal metabolic processes. Muscle atrophy as a result of this protein catabolism may be correlated with locomotor function, with smaller non-jumping muscles preferentially used as a protein source during fasting over larger jumping muscles. Higher levels of endogenous antioxidants in the jumping muscles may confer a protective advantage against oxidative damage during aestivation; however, it is not clear whether they play a role during aestivation or upon resumption of normal metabolic activity.

Key words: aestivation, antioxidants, morphometrics, muscle disuse atrophy, myofibre, oxidative damage, skeletal muscle, *Cyclorana alboguttata*.

INTRODUCTION

Muscle disuse atrophy is characterised by a loss of whole muscle mass, muscle cross-sectional area (CSA) and diminished contractile and locomotor ability (Witzmann et al., 1982). The magnitude of muscle disuse atrophy is variable and depends upon the duration of disuse, the specific muscle affected and its fibre composition (Hudson and Franklin, 2002b). Most muscles are composed of a mixture of slow-twitch (oxidative) or fast-twitch (glycolytic) myofibres, although there are exceptions (e.g. Ariano et al., 1973). In general, slow-twitch (oxidative) fibres are more vulnerable to muscle atrophy than fast-twitch (glycolytic) fibres (Maier et al., 1972; Tomanek and Lund, 1974).

The metabolic profiles of oxidative and glycolytic myofibres are inextricably linked to muscle disuse atrophy because oxidative stress is thought to contribute to atrophy in mammalian muscle (Kondo et al., 1991; Kondo et al., 1993; Kondo et al., 1994). Reactive oxygen species (ROS) are a natural by-product of oxidative metabolism, and in a normal metabolic environment there is a balance between the production of ROS and the protective antioxidants that scavenge or deactivate them (Ames et al., 1993). During immobilisation or dormancy, ROS production can outweigh the endogenous antioxidants, leading to oxidative stress, cell damage and atrophy (Kondo et al., 1991; Kondo et al., 1994). The metabolic profiles of myofibres may change during prolonged immobilisation with oxidative fibres becoming more like glycolytic fibres (Diffee et al., 1991; Roy et al., 1996; Talmadge, 2000; Pierno et al., 2002; D'Antona et al., 2003).

Animals that experience regular bouts of dormancy, such as hibernating mammals, provide a fascinating model for investigating muscle atrophy associated with disuse. During dormancy the animal exhibits a decline in metabolic and locomotor activity and become reliant upon stored lipids, with protein meeting additional energy demands (Carey et al., 2003). Like hibernation, amphibian aestivation occurs in response to unfavourable conditions, specifically a dry and warm (>10°C) environment and dwindling food resources, and is characterised by the construction of a burrow, the adoption of a water conserving posture and a metabolic depression (Pinder et al., 1992). The green-striped burrowing frog *Cyclorana alboguttata* (Günther 1867) (Meyer et al., 1997) is an amphibian aestivator that inhabits the arid and semi-arid zones of eastern Australia. During aestivation, these frogs are immobilised in a cast-like cocoon made from sloughed skin and are completely reliant upon stored lipids for metabolism (van Beurden, 1980).

Few studies have explored the effect of dormancy on muscle disuse atrophy in skeletal muscle (for reviews, see Carey et al., 2003; Shavlakadze and Grounds, 2006). In general, hibernators exhibit some level of muscle atrophy, although to a lesser extent than would be observed in humans under the same conditions (Yacoe, 1983; Wickler et al., 1987; Steffen et al., 1991; Wickler et al., 1991). There appear to be no published studies that examine the relationship between muscle fibre composition and biomechanical function and how this relationship might influence muscle disuse atrophy. Hudson and Franklin were the first to show that *C. alboguttata*

maintained whole muscle mass and contractile performance in several hindlimb muscles after 12 weeks of immobilisation during aestivation (Hudson and Franklin, 2002a). Recently, we found that the morphology and contractile performance of two hindlimb muscles (sartorius and iliofibularis) of *C. alboguttata* remained unchanged from that of active frogs after nine months of aestivation, suggesting an absence of muscle disuse atrophy despite prolonged disuse (Symonds et al., 2007).

In anurans, the muscles that provide power for jumping, such as the gastrocnemius and cruralis, are generally acknowledged as being predominantly glycolytic (Marsh, 1994). The iliofibularis and sartorius muscles, however, are described as being predominantly oxidative (Lutz et al., 1998), and are not involved in producing power jumps during locomotion (Calow and Alexander, 1973; Lutz et al., 1998). The aim of the present study was to examine the effect of prolonged aestivation on structural and biochemical characteristics of whole muscles from *C. alboguttata*. We hypothesised that muscle disuse atrophy would vary depending upon the myofibre composition, in conjunction with the biomechanical function, of the muscle. We proposed that smaller non-jumping muscles would be more susceptible to atrophy during disuse than larger jumping muscles, and that the changes caused by aestivation would increase with aestivation duration. We measured the effect of aestivation on whole muscle CSA, myofibre type, myofibre number and myofibre CSA. By comparing the myofibre composition of muscles throughout aestivation, we determined whether myofibre transition was occurring, with oxidative fibres being replaced by atrophy-resistant glycolytic myofibres. We measured total antioxidant power (TAP) in each muscle type to establish whether endogenous antioxidants were bolstered during aestivation to provide a protective function against oxidative stress. We compared muscle structure and biochemistry after six months and nine months of aestivation to clarify whether any changes associated with disuse were time dependent.

MATERIALS AND METHODS

Animal husbandry

Mature green-striped burrowing frogs *Cyclorana alboguttata* were collected from roadsides after heavy rain from Lake Broadwater, Darling Downs, SW Queensland, Australia. Individuals were transported to the laboratory within 24 h of capture in plastic bags and were sexed. Body mass was recorded using an electronic balance (BP310S, Sartorius, Edgewood, NY, USA) to the nearest 0.01 g, and snout–vent length (SVL) was measured using digital vernier callipers (Whitworth, Brisbane, Australia) to the nearest 0.01 mm. Each frog was randomly assigned to either an active group or to one of two aestivation groups: six-months and nine-months aestivation. There was no significant difference in initial frog size among groups as measured by SVL (mm) [one-way analysis of variance (ANOVA); $F_{2,33}$=0.0134, P=0.987)]. Active frogs were housed individually in 4 litre plastic containers with a moist paper towel substrate and water to a depth of 10 mm. The water and paper towel were replaced once per week, and frogs were fed *ad libitum* on house crickets and wood cockroaches dusted in Reptivite© vitamin supplement (Zoo Med, San Luis Obispo, CA, USA). Prior to aestivation, the treatment frogs were fed as per the control frogs for a period of four weeks. To induce aestivation, each treatment frog was placed into a 4 litre plastic ice-cream container filled with wet clay collected from the frogs' natural habitat. In most cases, the treatment frogs burrowed immediately, and the clay was permitted to dry slowly. Individuals were checked daily to ensure they remained burrowed. Any treatment individual that did not burrow into the clay after a period of three consecutive days was removed from the treatment group and replaced. All frogs were maintained in the facility at a constant temperature of 23°C and 12 h:12 h light:dark cycle. At the conclusion of the aestivation period (six- or nine-months) the clay block was broken open and the frog was extracted and immediately euthanised by cranial and spinal pithing. Control frogs were pithed at the start of the experimental period (i.e. when all frogs were deemed to be in aestivation), as well as at six- and nine-months. Body mass and SVL were re-measured post-euthanasia.

Dissection

Eight muscles were chosen for the measurement of whole muscle mass: six hindlimb muscles (gastrocnemius, cruralis, sartorius, semimembranosis, gracilis major and iliofibularis), an abdominal muscle (rectus abdominus) and a forelimb muscle (deltoideus). Each muscle was extracted from the frog immediately after pithing. Wet muscle mass was recorded and size-corrected using SVL (mm). The dry mass and water content of each muscle was determined by drying to a constant mass in a 60°C oven and size-corrected using SVL (mm).

Histochemistry

Four locomotor muscles were selected for histochemical analysis. Cross-sectional slices of approximately 5 mm thickness were taken from the mid-section of the sartorius, iliofibularis, cruralis and gastrocnemius muscles. The fresh muscle slices were placed into plastic moulds, mounted in Tissue-Tek™ OCT compound (ProSciTech, Townsville, Australia) and plunged into isopentane (2-methylbutane), cooled to –150°C in liquid nitrogen for approximately 30 s. The frozen blocks were removed and wrapped in aluminium foil to prevent desiccation and stored at –80°C in an air-tight container prior to sectioning.

Whole muscle and individual fibre morphometrics were determined by histochemical analysis as per Symonds et al. (Symonds et al., 2007). In brief, frozen 10 μm tissue sections were melted directly onto glass slides and stained immediately for succinic dehydrogenase (SDH) activity to differentiate between oxidative and glycolytic myofibres. One serial section from each muscle was photographed at a ×250 magnification with a digital camera (DFC280, Leica, Solms, Germany) mounted to a compound light microscope (BH2, Olympus, Mt Waverly, Victoria, Australia). Digital images were analysed with SigmaScan™ (SPSS Inc., Chicago, IL, USA) to determine muscle CSA, fibre area, fibre number and fibre density of each whole muscle section.

Antioxidant biochemistry

Four locomotor muscles were chosen for measurement of TAP: cruralis, gastrocnemius, sartorius and iliofibularis. Each muscle was the contralateral equivalent of the muscle used for the histochemical analysis. After extraction, the muscle was sliced, snap-frozen in liquid nitrogen and stored at –80°C until required. Prior to analysis the muscle was permitted to thaw on ice and was minced on an ice-cold cutting board with a single-edged razor blade. Approximately 0.2 g of tissue was homogenised with a bead beater (BIO101 FastPrep FP120, Vista, CA, USA) in 1000 μl of PBS in two 30 s pulses. Between pulses the sample was kept on ice for 2 min. The homogenate was transferred into a fresh Eppendorf tube and spun at 3000 ***g*** in a benchtop microcentrifuge (Clements Orbital 460, North Sydney, New South Wales, Australia) for 3 min at 4°C. The supernatant was divided into aliquots and stored at –80°C prior to analysis of water-soluble proteins.

TAP of water-soluble antioxidants (e.g. uric acid, vitamin C, bilirubin, thiols and glutathione) was determined using a

commercially available colorimetric kit (Total Antioxidant Power; Oxford Biomedical Research, BioNovus Life Sciences, Cherrybrook, New South Wales, Australia) following the manufacturer's instructions. Briefly, the TAP in a muscle tissue sample was detected by the evaluation of Cu^{+} derived from Cu^{2+} by the combined action of all of the antioxidants present in the sample. The Cu^{+} thus generated is detected following the complex formation between Cu^{+} and bathocuproine (BC), which has an absorption maximum of between 480 nm and 490 nm. The colorimetric reading was assayed on a multiplate spectrophotometer at 490 nm. Total protein content was determined using a commercially available spectrophotometric kit (BSA protein assay, Sigma Chemical Co., St Louis, MO, USA) and assayed at 562 nm. The TAP results are expressed per microgram of total protein.

Statistical analyses

All data are presented as means ± s.e.m. Data were standardised to a frog with SVL of 50 mm, with the exception of whole muscle dry mass (g) and water content (%), which is given as raw values, and whole muscle CSA (μm^2), which is indexed over individual frog SVL (mm). Muscle dry mass, water content, whole muscle CSA and mean myofibre number were analysed by one-way analysis of variance (ANOVA). Dry muscle mass, muscle water content and mean myofibre area were analysed by two-way ANOVA, with treatment group and muscle type or treatment group and fibre type as the factors for the analysis, respectively. Where differences were detected they were localised by a Holm–Sidak multiple comparison test. Assumptions of normality or equality of variance were violated for three data sets (whole muscle CSA only) and the data were $\log_{10}$-transformed. Transformed data were analysed using Kruskal–Wallis ANOVA on ranks and Dunn's multiple comparison procedure. All statistical analyses were performed with the statistical program SigmaStat™. In all cases P=0.05 was accepted for statistical significance.

RESULTS

Muscle morphology

There was no significant difference in dry muscle mass (g) among the treatment groups for the gastrocnemius, gracilis major, semimembranosis, cruralis, rectus abdominus and deltoideus muscles (Fig. 1). There was a significant decrease in dry muscle mass of the sartorius between controls and nine-month aestivators (*post-hoc* P=0.042), and there were significant decreases in dry muscle masses among all treatment groups for the iliofibularis ($F_{2,19}$=14.971, P<0.05) (Fig. 1). Muscle water content (% of wet muscle mass) remained unchanged throughout aestivation, except for the sartorius, which showed a significant increase in water content after six months and nine months of aestivation ($F_{2,19}$=7.013, P=0.006) (Fig. 2).

There was a significant decrease in CSA of the iliofibularis by 50% ($F_{2,22}$=7.991, P=0.003) (Fig. 3A) and the gastrocnemius by 30% ($F_{2,16}$=5.535, P=0.017) (Fig. 3C) after six months of aestivation; however, the CSA stabilised and was unchanged after nine months of aestivation. The CSA of the sartorius muscle remained unchanged after six months of aestivation but significantly decreased by 57% after nine months ($F_{2,22}$=4.522, P=0.024) (Fig. 3B). There was no significant difference in the CSA of the cruralis muscle between the treatment groups (Fig. 3D).

Fibre morphology

There were no significant differences in mean fibre number (per mm^2) among treatment groups for any of the muscles examined in this study (Fig. 4A–D). There was a significant difference in fibre type between muscles ($F_{3,21}$=4.698, P=0.014) with the iliofibularis having a significantly higher percentage of oxidative fibres at control (P=0.014 and nine-month (P=0.005) aestivation time points, and a significantly lower percentage of glycolytic fibres at control (P=0.014) and nine-month (P=0.005) aestivation time points (Table 1).

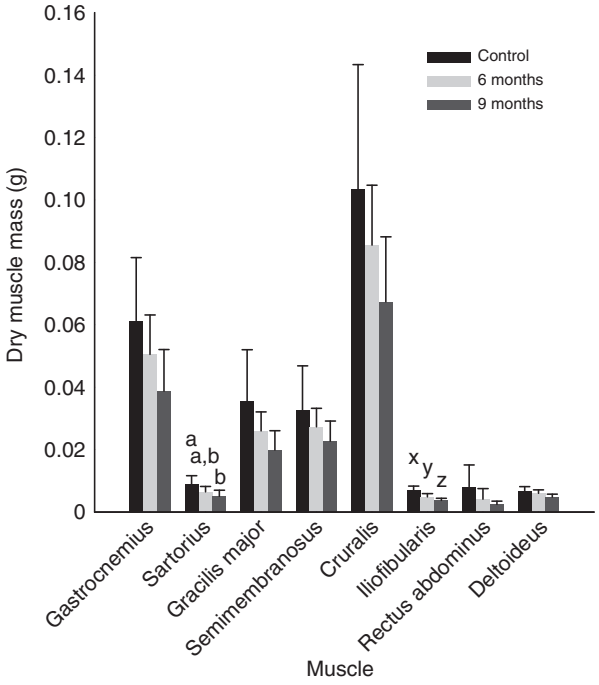


Fig. 1. Dry muscle mass (g) of eight skeletal muscles from active and aestivating *Cyclorana alboguttata* (N=10). The letters a and b, and x, y and z indicate significantly different data sets. Values are means ± s.e.m.

There was no significant difference in the CSA of oxidative or glycolytic myofibres among treatment groups in the sartorius (Fig. 5B) and gastrocnemius muscles (Fig. 5C). Iliofibularis myofibre CSA was significantly different among treatment groups for both myofibre types ($F_{2,14}$=26.162, P<0.001) (Fig. 5A). Iliofibularis oxidative myofibre CSA decreased by 42% after six months of aestivation and no further change after nine months, and glycolytic myofibre CSA decreased by 37% after six months of aestivation and no further change after nine months. Cruralis myofibre CSA was significantly different among treatment groups for both myofibre types ($F_{2,15}$=4.605, P=0.031) (Fig. 5D). Cruralis oxidative myofibre CSA decreased by 26% after six months of aestivation, while glycolytic myofibre CSA decreased by 22% after six months of aestivation. There were no further changes in these parameters after nine months of aestivation.

A summary of results from the morphological analysis is presented in Table 2.

Antioxidant biochemistry

There were no significant differences between control and six-month aestivation treatment groups in TAP for any of the muscles examined (Fig. 6). Following Hudson et al. (Hudson et al., 2006), we normalised the aestivator TAP data to actual oxygen consumption, on the basis that resting metabolic rate in aestivating Cycloranids is depressed by 70–80% (van Beurden, 1980; Withers, 1993). Using a conservative estimate of a 70% depression in whole animal metabolic rate, normalised TAP in six-month aestivators is

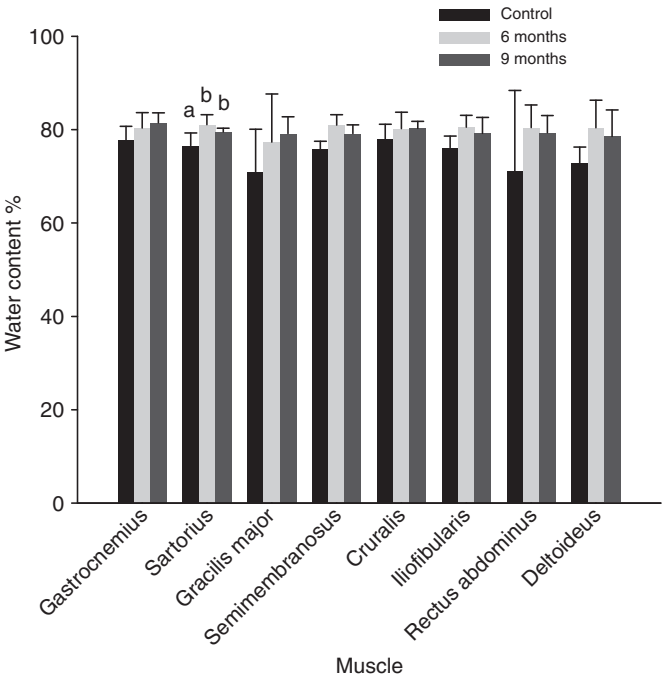


Fig. 2. Water content (% of wet muscle mass) of eight skeletal muscles from active and aestivating *Cyclorana alboguttata*. The letters a and b indicate significantly different data sets. Values are means ± s.e.m.

upregulated by 434% in the gastrocnemius, 252% in the cruralis, 186% in the iliofibularis and 139% in the sartorius (Fig. 6).

DISCUSSION

The discussion that follows is based on the assumption that limb immobilisation in ectotherms is a driving feature of the muscle phenotypes observed in aestivating frogs, but it is probably true that these observations are influenced to some extent by fasting (McDonagh et al., 2004). The results from this study show that prolonged aestivation of six to nine months duration produced morphological changes in selected skeletal muscles of *C. alboguttata* (Table 2). Powerful jumping muscles, such as the cruralis and gastrocnemius, appeared to be less susceptible to atrophic effects of prolonged immobilisation compared with the smaller non-jumping muscles, such as the sartorius and iliofibularis. Whole muscle mass, muscle CSA, water content and myofibre number remained unchanged in the cruralis muscle after six to nine months of aestivation. Similarly, whole muscle mass, water content, myofibre number and myofibre CSA remained unchanged in the gastrocnemius muscle after six to nine months of aestivation. However, iliofibularis muscle CSA was reduced by 30% and myofibre CSA was reduced by 42%. Protective endogenous antioxidants were functionally upregulated in all four muscles examined; however, the greatest increases in TAP occurred in the powerful jumping muscles. These results suggest that *C. alboguttata* may selectively preserve jumping muscles at the expense of non-jumping muscles in order to maintain life and locomotory capacity simultaneously during aestivation.

Muscle morphology

Dry muscle mass was unchanged throughout aestivation for all of the muscles examined in this study, except for the iliofibularis ($P<0.05$) and sartorius ($P=0.042$) (Fig. 1). The conservation of dry muscle mass indicates preservation of the muscle tissue, and hence muscle protein, of *C. alboguttata* throughout the aestivation period. The significant decreases in dry muscle mass of the iliofibularis and sartorius muscles is interesting given that both muscles are reported in the literature as being predominantly 'oxidative' (Lutz et al., 1998) and are not involved in producing power jumps during locomotion (Calow and Alexander, 1973; Lutz et al., 1998). This suggests that at a particular time point in aestivation (e.g. six- or nine-months) lipid reserves are depleted, forcing the frog to utilise other sources

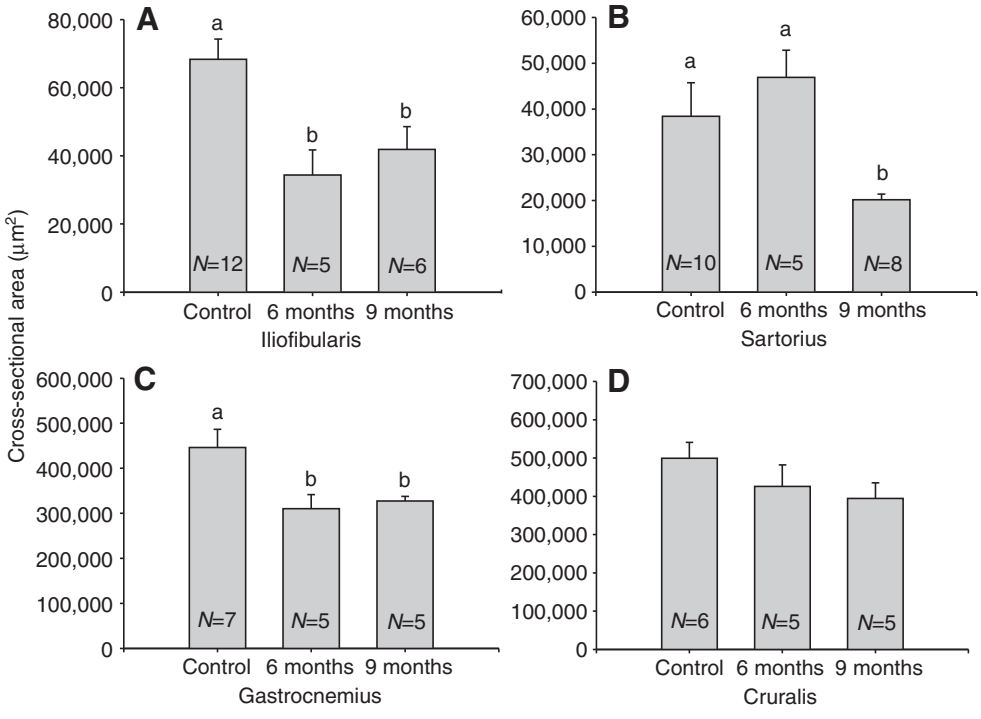


Fig. 3. Whole muscle cross-sectional area (μm²) of (A) iliofibularis, (B) sartorius, (C) gastrocnemius and (D) cruralis muscles from control (active) and aestivating *Cyclorana alboguttata*, standardised over frog snout–vent length (SVL) (mm). The letters a and b indicate significantly different data sets. Values are means ± s.e.m.

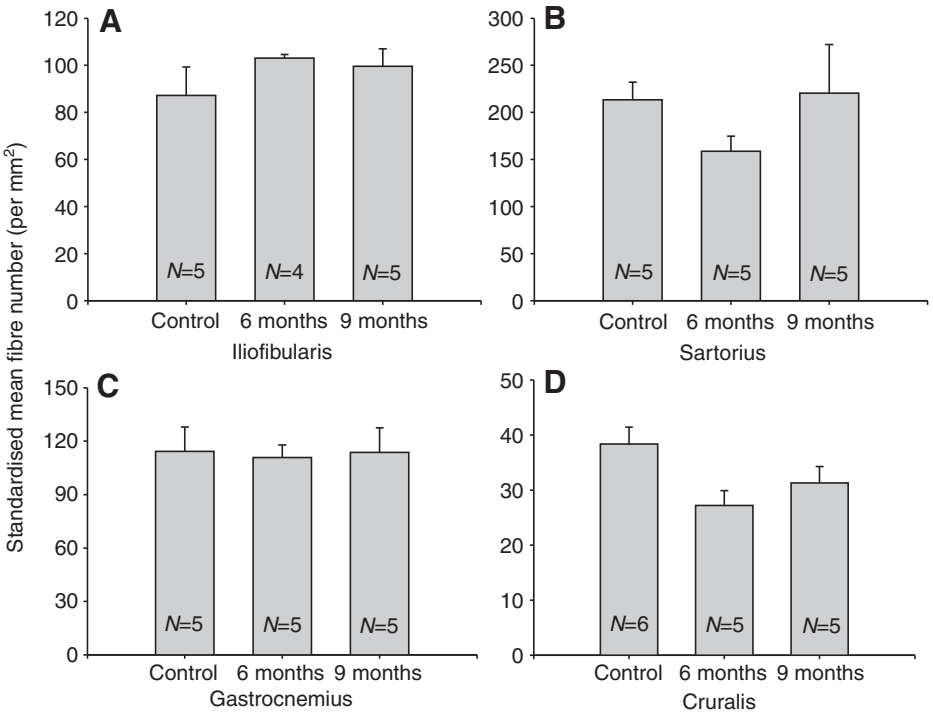


Fig. 4. Total mean myofibre number (per mm^2) of the (A) iliofibularis, (B) sartorius, (C) gastrocnemius and (D) cruralis from control (active) and aestivating *Cyclorana alboguttata*. Fibre counts were standardised to a frog with snout–vent length (SVL)=50 mm. Values are means ± s.e.m.

of fuel for maintaining metabolic processes. After liver glycogen and abdominal adipose reserves, skeletal muscle protein becomes the fuel for maintaining life during prolonged fasting, whereupon 'selective atrophy' of skeletal muscles that are not critical for prey-capture or predator escape would be beneficial (van Beurden, 1980). No other studies on muscle disuse atrophy in hibernating or aestivating animals have addressed the question of whether sequential atrophy occurs in relation to muscle function.

Skeletal muscle function is correlated with myofibre composition; therefore, it was expected that the non-power-producing muscles examined in this study would contain a higher proportion of oxidative myofibres than the jumping muscles. Our study demonstrated that the iliofibularis of *C. alboguttata* contained significantly more oxidative fibres than any other muscle examined (P=0.014), while the sartorius contained significantly more oxidative fibres than the cruralis but less than the gastrocnemius (P=0.014) (Table 1). Taken together with locomotory function, this result may support the hypothesis that oxidative muscles in amphibians are more susceptible to muscle disuse atrophy than glycolytic muscles, as is the case for mammals (Maier et al., 1972; Tomanek and Lund, 1974).

The water contents of all of the muscles from *C. alboguttata* were maintained at control levels throughout aestivation, with the exception of the sartorius, in which water content significantly increased (Fig. 2). The conservation of muscle water content throughout aestivation attests to the ability of this species to actively protect body tissues against dehydration (Withers, 1998). There is a strong relationship between muscle water content and muscle disuse atrophy, linked by the effect of starvation on muscle enzymes, and it is clear that *C. alboguttata* fast during aestivation (Hudson et al., 2006; Cramp et al., 2005). It has previously been shown in Atlantic cod (*Gadus morhua*) that an increase in muscle water content from 79% to 92% resulted in a 10-fold decrease in enzyme activity levels (Lemieux et al., 2004). Given that there is a significant increase in the water content of the sartorius, it is possible that an increase in muscle water content is indicative of down-regulation of enzyme activity during dormancy. A down-regulation of muscle enzymes in the relatively non-essential sartorius muscle during

Table 1. Relative proportions of each myofibre type (% area) contributing to each muscle from active and aestivating *Cyclorana alboguttata* (N=6)

Myofibre types %	Control	Six-month aestivators	Nine-month aestivators	P-value
Oxidative				
Iliofibularis	32.95±1.60	31.98±2.74	31.52±1.45	0.846
Sartorius	26.95±2.21	29.29±2.34	28.78±0.91	0.676
Gastrocnemius	29.52±0.89	33.11±4.30	29.51±0.42	0.990
Cruralis	26.08±0.88	25.02±1.18	25.06±1.19	0.724
P-value	0.014*	0.235	0.005*	
Glycolytic				
Iliofibularis	67.05±1.60	68.20±2.74	68.48±1.45	0.846
Sartorius	73.05±2.21	70.71±2.34	71.22±0.91	0.676
Gastrocnemius	70.47±0.89	66.89±4.30	70.49±0.42	0.990
Cruralis	73.92±0.88	74.98±1.18	74.94±1.18	0.724
P-value	0.014*	0.235	0.005*	

Values are means ± s.e.m. *Indicate significant differences.

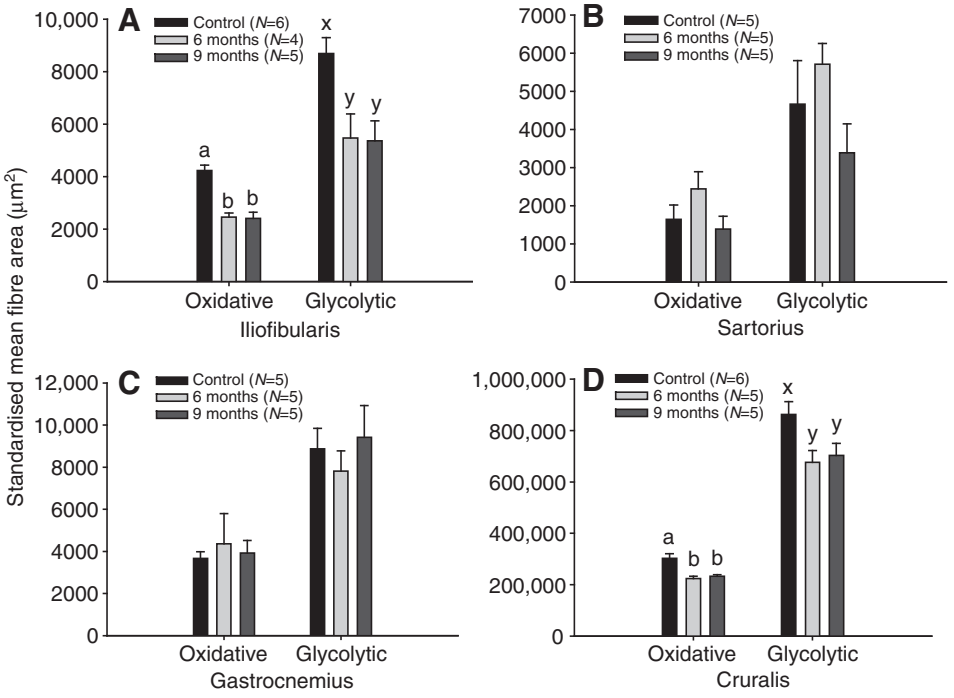


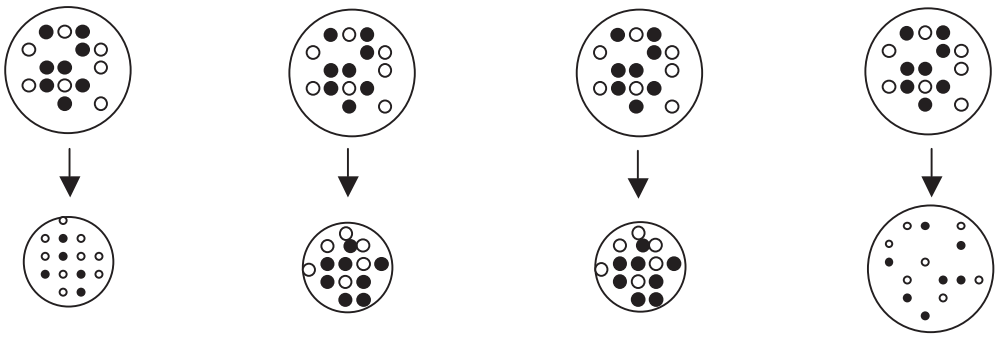
Fig. 5. Mean oxidative and glycolytic myofibre area (μm^2) of the (A) iliofibularis, (B) sartorius, (C) gastrocnemius and (D) cruralis from control (active) and aestivating *Cyclorana alboguttata* (*N*=5 for all groups). Myofibre area was standardised to a frog with snout–vent length (SVL)=50 mm. The letters a and b, and x and y indicate significantly different data sets. Values are means ± s.e.m.

dormancy would imply a reduced metabolic demand on the tissue thereby potentially prolonging survival on endogenous fuel supplies without compromising the animals' locomotory function upon arousal. This may explain why the sartorius is less affected by prolonged aestivation.

Prolonged aestivation resulted in significant decreases in whole muscle CSA in three out of four muscles examined in this study. Gastrocnemius CSA decreased by 30% (Fig. 3C), and the CSA of the iliofibularis muscle decreased by 50% after six months of aestivation (Fig. 3A). The sartorius muscle from six-month aestivators maintained CSA at control levels and then significantly decreased by 22% after nine months of aestivation (Fig. 3B). These reductions in whole muscle CSA and mass are significant and they are not dissimilar to previous findings on hibernating mammals, which range from 14% to 63% (Steffen et al., 1991; Yacoe, 1983; Wickler et al., 1987; Reid et al., 1995; Wickler et al., 1991). The cruralis muscle, the largest of the four muscles examined and a powerful jumping muscle, did not exhibit any change in CSA during aestivation (Fig. 3D). Taking into account the comparatively high body temperature of aestivating *C. alboguttata* (approximately 25°C)

Table 2. Summary of statistically significant morphological changes observed in aestivating *Cyclorana alboguttata*

	Iliofibularis	Sartorius	Gastrocnemius	Cruralis
Muscle functions (from Duellman and Trueb, 1994)	Most oxidative Non-jumping muscle Flexes the knee; abducts the femur	Moderately oxidative Non-jumping muscle Abducts the femur; flexes the knee	Moderately oxidative Jumping muscle Straightens the ankle joint	Least oxidative Jumping muscle Extends knee joint; flexes the hip joint
Dry muscle mass (g)	Decreased	Decreased	n.s.	n.s.
Water content (%)	n.s.	Increased	n.s.	n.s.
Whole muscle CSA (mm)	Decreased at six months	Decreased at nine months	Decreased at six months	n.s.
Oxidative myofibre area (μm^2)	Decreased at six months	n.s.	n.s.	Decreased at six months
Glycolytic myofibre area (μm^2)	Decreased at six months	n.s.	n.s.	Decreased at six months



n.s. refers to non-significant differences.

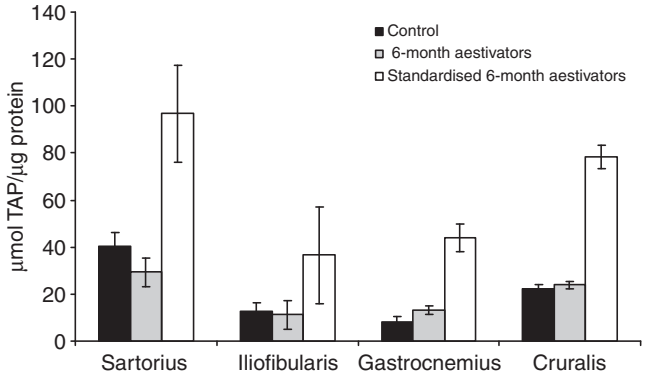


Fig. 6. Total antioxidant power (µmol TAP µg^{-1} protein) from the iliofibularis, sartorius, gastrocnmeius and cruralis muscles of control (active) and aestivating *Cyclorana alboguttata*. Data from the six-month aestivators have also been normalised to resting metabolic rate (=oxygen consumption) during aestivation.

and the long period of dormancy (nine months), the structural maintenance (dry mass, water content and muscle CSA) of this muscle is remarkable.

Previous studies have shown that immobilisation and unloading can result in a transition from slow to fast myofibres (Roy et al., 1996; Pierno et al., 2002). The transition occurs when the myofibres shift towards a myosin isoform with higher ATPase activity (Diffee et al., 1991). Regulation of the pathways responsible for myofibre transition are complex, and little is known about the signalling mechanisms that translate changes in neuromuscular activity into myosin isoform remodelling (Talmadge, 2000). However, a consequence of this transition is that the affected muscles produce more force at any shortening velocity compared with unaffected muscles and hence preserve maximal power output despite muscle atrophy (Caiozzo, 2002). It is possible that the observed changes in muscle CSA in *C. alboguttata* are due to preferential atrophy of oxidative myofibres and thus compromise the animal's capacity for endurance activity.

Muscle CSA is positively correlated with peak force production; therefore, a consequence of muscle atrophy is diminished locomotory ability (Booth and Kelso, 1973). Compromised locomotion may impact on many factors that affect survival, including predator escape, prey capture and reproduction. We have previously shown that *C. alboguttata* is able to preserve locomotory performance, as measured by power output (W kg^{-1}) and maximal twitch force production (nmol mm^{-2}), after nine months of aestivation despite some changes to muscle morphology (Symonds et al., 2007; Hudson et al., 2006). This outcome is consistent with a biological system in which protein is selectively catabolised from specific muscles in order to maintain life during prolonged dormancy. We propose that *C. alboguttata* maintains locomotor function in the face of a small amount of muscle atrophy and the remaining skeletal muscle is highly preserved (Hudson and Franklin, 2002a; Hudson and Franklin, 2002b). In other words, *C. alboguttata* may take a 'quality not quantity' approach to balancing the maintenance of basal metabolism with preserving locomotory ability.

Fibre morphology

There were no significant differences between treatment groups in the total number of myofibres present within each muscle examined (Fig. 4A–D), indicating that loss of myofibres (hypoplasia) was not a factor contributing to the observed changes in whole muscle CSA. Myofibre CSA significantly decreased in the iliofibularis and cruralis muscles but was preserved in the sartorius and gastrocnemius muscles during prolonged aestivation (Fig. 5A–D). Myofibre atrophy occurred rapidly in the iliofibularis (within the first six months of aestivation), which is consistent with the hypothesis that oxidative muscles are more susceptible to muscle atrophy during disuse. These data are consistent with the reduction in whole muscle dry mass (Fig. 1) and whole muscle CSA (Fig. 3) observed in the iliofibularis during aestivation. The reduction in cruralis myofibre CSA is more surprising given that there were no observed changes to whole muscle dry mass (Fig. 1) or CSA (Fig. 3). It is worth noting that there was a non-significant trend towards decreasing whole muscle CSA in the cruralis, which could be explained by the significant reduction in myofibre CSA.

Lutz et al. (Lutz et al., 1998) found that the iliofibularis and sartorius muscles *Rana pipiens* were strongly oxidative (52% and 37% of total CSA, respectively) compared with the cruralis and gastrocnemius (10% and 11% of total CSA, respectively). Remarkably, the relative proportions of myofibre type in *C. alboguttata* were very similar among muscles and treatment groups (Table 1). Although significant differences were detected among control muscles and nine-month aestivator muscles, the proportions of oxidative fibres present within each muscle were confined within a narrow range of 25–33%. Previously, this pattern of homogenous myofibre composition has only been observed in one other animal, the Etruscan shrew *Suncus etruscus* (Jurgens, 2002). This homogeneity may be an adaptive measure geared towards lowering the overall metabolic demands of the skeletal muscles and extending fuel reserves during aestivation.

Antioxidant biochemistry

Our results demonstrate that protective endogenous antioxidants are maintained at control levels in aestivating *C. alboguttata* despite a substantial metabolic depression of approximately 70% (Fig. 6). Animals that are susceptible to oxidative stress during dormancy may maintain high levels of antioxidant defences either permanently, e.g. freshwater turtles (Storey, 1996; Willmore and Storey, 1997), or when needed in anticipation of an onslaught of free radicals, e.g. land snails (Hermes-Lima et al., 1998). When TAP is expressed in proportion to whole animal metabolic rate during aestivation, it is apparent that antioxidant production is increased by up to 434% during aestivation. This suggests that antioxidant defenses are modulated in response to the rate of production of ROS, which is proportional to oxygen consumption (Grundy and Storey, 1998). The results from this study are similar to the findings of previous studies, in which chipmunks (Fukuhara et al., 2006), snails (Ferreira et al., 2003) and frogs (Hudson et al., 2006) modulated endogenous antioxidant production during hibernation to defend organs and tissues against oxidative stress.

The present study shows that the protection offered by endogenous antioxidants against muscle disuse atrophy is not unassailable as shown by the non-jumping muscles, the iliofibularis and the sartorius, which exhibited significant levels of muscle disuse atrophy during aestivation (Figs 1 and 3). Conversely, TAP was relatively higher in the gastrocnemius and cruralis muscles (i.e. the powerful jumping muscles) than the iliofibularis and sartorius muscles (Fig. 6), supporting the hypothesis that endogenous antioxidants may play a role in protecting the muscles against damage caused by oxidative stress. If true, this does not explain the decrease in gastrocnemius whole muscle CSA (Fig. 3) and the decline in cruralis myofibre CSA (Fig. 5). Furthermore, the

histological data suggests there is very little metabolic difference among the four muscles examined in this study (Fig. 6). However, antioxidant production can only account for some attenuation of oxidative damage, just as oxidant production only accounts for a proportion of the atrophy process, and it is possible that the observed atrophy may have been even more severe in the absence of antioxidant defences.

An alternative explanation for the role of endogenous antioxidants in aestivating *C. alboguttata* involves preparing for oxidative stress with the resumption of normal oxidative metabolism (Ferreira et al., 2003; Hermes-Lima and Storey, 1998; Hermes-Lima and Zenteno-Savin, 2002). Rather than 'inhibiting' muscle disuse atrophy in *C. alboguttata* during aestivation, the protective role of endogenous antioxidants may begin upon arousal. The production of damaging ROS is likely to be extremely low or even negligible during the metabolic depression observed during aestivation, which supports this hypothesis.

In this way, metabolic depression can play a role in protecting animals from oxidative stress (Hudson and Franklin, 2002b). The reduction in oxygen consumption and oxidative metabolism simultaneously reduces the production of ROS (Adelman et al., 1988), suggesting that the extreme metabolic depression observed in *C. alboguttata* is associated with limiting ROS production and hence muscle disuse atrophy. However, during aestivation *C. alboguttata*, like other aestivating anurans, is primarily reliant on energy that is stored in large lipid bodies (Jones, 1980). While this negates immediate utilisation of muscle protein as an energy substrate, accessing the energy stored as lipids occurs *via* peroxidation, a process that liberates large quantities of damaging ROS in the form of peroxide (H_2O_2) (Moylan and Reid, 2007; Grundy and Storey, 1998). As such, the energy pathway used during dormancy to maintain basic life processes may outweigh any benefit conferred by metabolic depression on reducing oxidative stress in this species. Furthermore, it should be considered that while low, ROS production during aestivation is sustained over a long duration, which may still render the muscle susceptible to oxidative damage. The trade-off between hypometabolism and ROS production lends support to hypothesis that production of endogenous antioxidants during aestivation is a critical strategy for protecting important tissues, such as jumping muscles.

Concluding remarks

It is clear that *C. alboguttata* does not exhibit a typical atrophic response to prolonged immobilisation during dormancy. The muscle disuse atrophy observed was comparable with mammalian models of disuse, despite the longer timeframe and higher body temperature (in comparison with mammalian hibernators) experienced by this species. The mechanisms underlying the ability of this species to delegate and regulate protein catabolism based on muscle function are not yet known, and further investigation is required to determine the exact role of upregulating endogenous antioxidants during dormancy. The apparent metabolic homogeneity of the four muscles examined in this study, as shown by the enzyme histochemistry data, needs to be further investigated. Overall, further research is required to isolate the physiological, biochemical and molecular systems that operate to selectively preserve muscle tissue and maintain life during prolonged aestivation.

This project was funded by an ARC Discovery Grant awarded to C.E.F. B.L.M. was supported by a University of Queensland Mid-Year Scholarship. The authors would like to thank Lina Daddow for technical assistance. The experimental procedures were in full compliance with AEC (#SIB/572/06/UQ) and EPA (#WISP03572406) regulations. B.L.M. (nee Symonds) developed project design, acquired and analysed data, drafted the manuscript. N.J.H. contributed to project design, provided assistance with the acquisition and interpretation of TAP data and critical revision of manuscript draft. G.S.H. provided access to equipment and facilities needed for acquisition of TAP data. R.L.C. provided technical support and revised the manuscript. C.E.F. contributed to project design, provided financial and technical assistance with data acquisition and interpretation, and provided critical revision of manuscript draft.

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