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Getting the jump on skeletal muscle disuse atrophy: preservation of contractile performance in aestivating *Cyclorana alboguttata* (Günther 1867)

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Summary

Prolonged immobilisation or unloading of skeletal muscle causes muscle disuse atrophy, which is characterised by a reduction in muscle cross-sectional area and compromised locomotory function. Animals that enter seasonal dormancy, such as hibernators and aestivators, provide an interesting model for investigating atrophy associated with disuse. Previous research on the amphibian aestivator *Cyclorana alboguttata* (Günther 1867) demonstrated an absence of muscle disuse atrophy after 3 months of aestivation, as measured by gastrocnemius muscle contractile properties and locomotor performance. In this study, we aimed to investigate the effect of aestivation on iliofibularis and sartorius muscle morphology and contractile function of *C. alboguttata* over a longer, more ecologically relevant time-frame of 9 months. We found that whole muscle mass, muscle cross-sectional area, fibre number and proportions of fibre types remained unchanged after prolonged disuse. There was a significant reduction in iliofibularis fibre cross-sectional area (declined by 36% for oxidative fibre area and 39% for glycolytic fibre area) and sartorius fibre density (declined by 44%). Prolonged aestivation had little effect on the isometric properties of the skeletal muscle of *C. alboguttata*. There was a significant reduction in the isometric contraction times of the relatively slow-twitch iliofibularis muscle, suggesting that the muscle was becoming slower after 9 months of aestivation (time to peak twitch increased by 25%, time from peak twitch to half relaxation increased by 34% and time from last stimulus to half tetanus relation increased by 20%). However, the results of the work-loop analysis clearly demonstrate that, despite changes to muscle morphology and isometric kinetics, the overall contractile performance and power output levels of muscles from 9-month aestivating *C. alboguttata* are maintained at control levels.

Key words: aestivation, muscle disuse atrophy, fibre, morphology, contractile properties, locomotion, work loop, *Cyclorana alboguttata*.

Introduction

Skeletal muscle is a dynamic and plastic tissue that responds rapidly to changes in frequency and duration of use (Musacchia et al., 1988). An increase in muscle activity builds and maintains muscle mass, while inactivity results in tissue degeneration. In mammals, prolonged immobilisation or unloading of skeletal muscle results in muscle 'wasting' or muscle disuse atrophy (Booth, 1982). Muscle disuse atrophy is characterised by a reduction in muscle fibre cross-sectional area due to the combined effects of increased catabolism and decreased anabolism of skeletal muscle protein and an imbalance of muscle biochemistry (Kandarian and Stevenson, 2002).

In experimental models of hindlimb immobilisation and unloading the effects of muscle disuse atrophy on muscle morphology and muscle mechanics are variable and depend upon muscle type and fibre type (Ariano et al., 1973; Booth and Kelso, 1973; Edgerton et al., 2002; Peter et al., 1972; Trappe et al., 2004; Witzmann et al., 1982). Most muscles are composed of a heterogenous mixture of fibre types, although there are exceptions. For example, the mammalian soleus muscle is almost completely slow twitch (Ariano et al., 1973).

During normal activity, oxidative muscles tend to be used frequently, for extended periods of time and at low intensities. In comparison, fast-twitch glycolytic muscles are used infrequently, for short periods and at high intensity. When oxidative muscles become inactive the scope of the change in the level of activity is much greater than that experienced by glycolytic, fast-twitch muscles (Hudson and Franklin, 2002b). In general, slow-twitch (oxidative) fibres are more vulnerable to muscle disuse atrophy than fast-twitch (glycolytic) fibres (Edgerton et al., 2002; Maier et al., 1973; Nordstrom et al., 1995; Tomanek and Lund, 1974).

Prolonged disuse alters muscle fibre type characteristics, with oxidative fibres acquiring the structural, biochemical and

mechanical properties of glycolytic fibres (Anderson et al., 1999; Diffee et al., 1991). Generally, tetanic stress is reduced, maximum rate of shortening is elevated, and fatigue resistance is slightly reduced or unaffected (Booth and Seider, 1979a; Roy et al., 1991; Roy et al., 1996; Roy et al., 2002). Commonly, the contractile rates of oxidative muscles are increased after immobilisation leading to quicker twitch times that are more similar to glycolytic muscles (Anderson et al., 1999; Canon and Goubel, 1995; Witzmann et al., 1982). Additionally, due to the comparatively greater numbers of mitochondria contained within them, oxidative fibres produce comparatively larger quantities of reactive oxygen species as a byproduct of normal cell metabolism. Reactive oxygen species, or free radicals, are responsible for stochastically damaging cell architecture, including proteins and lipids, which leads to cellular apoptosis and hence muscle disuse atrophy (Kondo et al., 1993a; Kondo et al., 1993b; Kondo et al., 1993c; Kondo et al., 1994). During disuse the dramatic downregulation of activity in the oxidative muscle may trigger profound changes in the biochemical function of the muscle mitochondria.

Few studies have explored the effect of dormancy on muscle disuse atrophy of skeletal muscle (for a review, see Carey et al., 2003). Of the available literature, the majority investigate mammalian models, but virtually none have been conducted on amphibians. In general, the animals studied exhibit some muscle disuse atrophy, though to a lesser degree than observed in humans under similar conditions of immobility (Harlow et al., 2001; Tinker et al., 1998). For example, hibernation for 6 months caused significant atrophy in skeletal muscle from the golden-mantled ground squirrel (*Spermophilus lateralis*) as measured by whole muscle mass (Wickler et al., 1991b) and whole muscle cross-sectional area, with observable concurrent, but non-significant, declines in the protein content of these muscles (Steffen et al., 1991).

Animals that experience regular bouts of dormancy, such as hibernators and aestivators, exhibit a decline in metabolic and locomotor activity that is correlated with seasonal fluctuations in temperature and resource availability (Guppy and Withers, 1999). During dormancy the animal is primarily reliant upon stored lipids, with protein meeting additional energy demands (Harlow et al., 2001; Pakay et al., 2002; Pedler et al., 1996; Seymour, 1973; Tinker et al., 1998; van Beurden, 1980). Muscle comprises the largest protein store in the body, therefore catabolism of muscle tissue as an energy substrate during hibernation and aestivation may contribute to muscle disuse atrophy (Steffen et al., 1991; Wickler et al., 1987; Wickler et al., 1991b; Yacoe, 1983). Additionally, it has been demonstrated that protein synthesis is downregulated during dormancy, which may further compound the atrophic effects of dormancy on skeletal muscle (Frerichs et al., 1998; Fuery et al., 1998; Pakay et al., 2002). As such, hibernators and aestivators provide a fascinating model for investigating muscle atrophy associated with disuse (see review by Hudson and Franklin, 2002b).

Amphibian aestivation usually occurs in response to unfavourable environmental conditions (Kobelt and Linsenmair, 1995), specifically a dry and warm (>10°C) environment, and is characterised by the construction of a thermally stable burrow (van Beurden, 1980), the formation of a cocoon (Withers, 1995; Withers, 1998) and metabolic rate depression (Flanigan et al., 1991; 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. This species undergoes aestivation for many months (6–9 months on average) between seasonal rain events (Withers and Richards, 1995). After 3 months of aestivation locomotor performance, wet muscle mass of several hindlimb muscles and contractile properties of the gastrocnemius of *C. alboguttata* remained unchanged from that of control animals, indicating an absence of muscle disuse atrophy (Hudson and Franklin, 2002a). Long bone mechanical properties, capillary structure and gut function were also preserved during aestivation in *C. alboguttata* (Cramp and Franklin, 2005; Hudson et al., 2004; Hudson and Franklin, 2003).

It is clear that although *C. alboguttata* does not exhibit typical atrophic responses, we are still unsure as to what exemplifies a typical aestivator response to prolonged muscle disuse. The aim of the present study was to examine the effect of prolonged aestivation on the morphology and contractile performance of whole muscles from *C. alboguttata*. Unlike previous studies, this project investigated the effect of aestivation over a dramatically longer, more ecologically relevant, time-frame of 9 months. At the time of publication, no other study had examined the effect of aestivation or hibernation on any aspect of muscle physiology or biochemistry over such an extended period. Though Hudson and Franklin's study (Hudson and Franklin, 2002b) demonstrated that *C. alboguttata* did not exhibit muscle disuse atrophy after 3 months of aestivation, we felt that the results of that study were not indicative of the true capabilities of this animal as a model species.

We hypothesised that aestivation of 9 months duration would result in changes to the fibre-type composition of the sartorius (relatively fast-twitch, glycolytic) and iliofibularis (relatively slow-twitch, oxidative) muscles. Specifically, the iliofibularis muscle would exhibit a compositional change from the atrophy-susceptible oxidative fibre type to the more atrophy-resistant glycolytic fibre type. If evident, we proposed these changes in fibre type would affect muscle contractile performance of aestivating frogs; the slow-twitch iliofibularis muscle would exhibit the contractile properties of a fast-twitch muscle, and the fast-twitch sartorius would become faster still. We measured the effect of aestivation on whole muscle cross-sectional area, muscle fibre type, fibre number and fibre cross-sectional area, and related the observed ultrastructural changes (if any) to muscle mechanical performance as measured by isometric twitch and tetanus kinetics, work-loop power output and work-loop fatigue resistance.

Materials and methods

Animal husbandry

Adult green-striped burrowing frogs *Cyclorana alboguttata* (Günther, 1867) of both sexes were collected from roadsides after heavy rain at Lake Broadwater, SW Queensland, Australia, between February 2005 and January 2006. The frogs were transported to the School of Integrative Biology at University of Queensland and were sexed. Body mass was measured using an electronic balance (BP310S, Sartorius, Edgewood, NJ, USA) to the nearest 0.01 g and snout–vent length (*SVL*) measured using digital Vernier callipers (Whitworth, Brisbane, Australia) to the nearest 0.01 mm. Each frog was randomly assigned to either a control (active) or treatment (9-month aestivation) group. There was no significant difference in initial frog size between control and treatment groups, as measured by *SVL* (mm) (*t*-test; P=0.896). Control frogs (N=6) were housed individually in 5 l plastic containers with a 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 crickets and wood cockroaches dusted with ReptiVite™ (ZooMed, San Luis Obispo, CA, USA) diet supplement. Treatment frogs (N=5) were similarly housed and fed for two weeks post-capture before being individually placed in a plastic container filled with wet clay (obtained from the field collection site) to a depth of 200 mm. Most individuals burrowed into the wet clay immediately, while any frog that did not burrow into the clay after 3 consecutive days was removed from the treatment group and replaced. The clay was permitted to dry naturally and the frogs began aestivating for 9 months. All frogs were kept in the facility at a constant 24°C under combined natural and fluorescent lighting conditions. At the conclusion of the 9-month treatment period, the treatment frogs were removed from the dry clay housing and immediately euthenised by cranial and spinal pithing. Control frogs were also pithed at this time. Body mass and *SVL* were re-measured post-euthanasia.

Dissection

The skin of both hind limbs was removed to expose the muscle tissue. Slices of approximately 5 mm thickness were dissected from the belly of the right-side iliofibularis and left-side sartorius muscles with a razor blade. The iliofibularis and sartorius were chosen to represent relatively slow and fast skeletal muscles, respectively, based upon previous studies of anuran muscle fibre type (Putnam and Bennett, 1983) and preliminary findings by the author. These 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 for histochemical analysis. The sartorius muscle from the right leg and the iliofibularis from the left leg of control and treatment frogs were removed, leaving a section of bone attached to the tendon at either end of each muscle. These muscles were stored in refrigerated McKenzies frog Ringer solution (111 mmol l^{-1} NaCl, 2.5 mmol l^{-1} KCl, 1.8 mmol l^{-1} $CaCl_2.2H_2O$, 1 mmol l^{-1} MgCl, 5 mmol l^{-1} Hepes, 10 mmol l^{-1} glucose, adjusted to pH 7.4 with NaOH) until required for isometric analysis.

Histochemistry

Whole muscle and individual fibre morphometrics were determined by histochemical analysis. Frozen muscle blocks were mounted onto stainless steel chucks with OCT compound, and placed in a cryostat chamber (HM505, Microm, Walldorf, Germany) at –20°C. Frozen cross sections (10 μm thickness) were cut and melted directly onto glass slides and air-dried for approximately 3 min. The sections were returned to the cryostat chamber for storage in an air-tight container prior to staining. Best results were achieved for muscle that was sectioned and stained on the same day. Slides were removed from the cryostat chamber and stained immediately for succinic dehydrogenase (SDH) activity to differentiate between oxidative and glycolytic muscle fibres: 50 mmol l^{-1} phosphate buffer, 80 mmol l^{-1} sodium succinate, 1 mg ml^{-1} Nitro blue tetrazolium, adjusted to pH 7.6 with NaOH (Rowlerson and Spurway, 1988). Sections were incubated in the staining medium in air-tight, light-proof containers for at least 90 min. Each slide was washed three times in distilled water, air dried and mounted with Crystal/Mount™ (Biomeda Corp., Burlingame, CA, USA). Each section was photographed at a 250× magnification with a digital camera (DFC280, Leica, Wetzlar, Germany) mounted to a compound light microscope (BH2, Olympus, LeHigh Valley, PA, USA). Digital images were analysed with SigmaScan™ (SPSS Inc., Chicago, IL, USA) to determine muscle cross-sectional area, fibre area, fibre number and fibre density of each whole muscle section.

Isometric analysis

Isometric analysis was used to determine the twitch and tetanus kinetics of the isolated hindlimb muscles. The bone at either end of the muscle preparation was clamped *via* crocodile clips to a strain gauge (UF1, Pioden Controls Ltd, Canterbury, UK) at one end, and a motor arm (V201, Ling Dynamics Systems, Royston, UK) attached to an LVDT (Linear Variable Displacement Transformer, DFG 5.0, Solartron Metrology, Bognor Regis, UK) at the other. The LVDT was used to monitor the length changes delivered to the muscle preparation. The whole of the muscle, tendon and bone preparation was then maintained within the organ bath at 25±0.5°C in circulating, oxygenated McKenzies frog Ringer solution. The preparation was held at constant length and stimulated *via* parallel platinum electrodes to generate a series of twitches. Stimulus amplitude, pulse width and muscle length were adjusted to determine the stimulation parameters and muscle length corresponding to maximal isometric twitch force. Time to peak twitch and time from peak twitch to half relaxation were measured *via* a Powerlab 4SP (AD Instruments, Colorado Springs, CO, USA) and viewed with Chart 5.0 software (AD Instruments). An isometric tetanic force response was elicited by subjecting the

muscle to a 300 ms train of stimulation. Stimulation frequency (130–150 Hz for iliofibularis; 140–170 Hz for sartorius) was altered to determine maximal tetanic force. Time to half peak tetanic force and time from last stimulus to half tetanic force relaxation were measured. A rest period of 5 min was allowed between each tetanic response.

Work loop analysis

The work-loop technique was used to determine the power output of muscles during cyclical length changes (Josephson, 1993). Unlike fixed-length isometric studies and fixed-load isotonic studies of muscle performance, the work-loop technique allows measures of muscle power output under length and activation changes that are generally more indicative of *in vivo* contractile performance (Caiozzo, 2002; James et al., 1996; Josephson, 1993). Each muscle preparation was subjected to a set of four sinusoidal length changes symmetrical around the length found to generate maximal twitch force. The muscle was stimulated using the stimulation amplitude and frequency found to yield maximal isometric force. Electrical stimulation and length changes were controlled *via* a data acquisition board and a custom-designed program developed with TestPoint software (Measurement Computing, Norton, MA, USA). Muscle force was plotted against muscle length for each cycle to generate a work loop, the area of which equated to the net work produced by the muscle during the cycle of length change (Josephson, 1993). The net work produced was multiplied by the frequency of length-change cycles to calculate net power output. The total strain of length-change cycles was maintained at 0.10 throughout all experiments, which equated to ± 5% of resting muscle length. The cycle frequency of length change was altered up and down within the range 1–9 Hz for iliofibularis and 2–10 Hz for sartorius to generate power output-cycle frequency curves. During these length change cycles the muscle was usually subjected to phasic stimulation (i.e. active work-loop cycle) but sometimes these length changes were performed without stimulation (passive work-loop cycle) to monitor the net work done on the muscle during the length change cycle. Every 5 min the muscle was subjected to a further set of four work-loop cycles with stimulation duration and stimulation phase parameters being altered until maximum net work was achieved at each cycle frequency. A set of control sinusoidal length change and stimulation parameters were imposed on the muscle every 3–4 sets of work loops to monitor variation in the muscle's ability to produce power/force. Any variation in power was found to be due to a matching change in ability to produce force. Therefore, the power produced by each preparation, prior to the fatigue run, was corrected to the control run that yielded the highest power output, assuming that alterations in power generating ability were linear over time. All muscles still produced over 80% of maximal control run power prior to the fatigue run. On completion of the power output-cycle frequency curve each muscle was subjected to a fatigue run consisting of 30 work-loop cycles at the lowest cycle frequency that had earlier been found to produce 80% of maximal power output.

At the end of the isometric and work-loop experiments, the bones and tendons were removed and each muscle was blotted on absorbent paper to remove excess Ringer solution. Wet muscle mass was determined to the nearest 0.0001 g using an electronic balance (BP211D, Sartorius). Mean muscle cross-sectional area was calculated from muscle length and mass assuming a density of 1060 kg m^{-3} (Mendez and Keys, 1960). Maximum isometric muscle stress was then calculated as maximum tetanic force divided by mean cross-sectional area (kN m^{-2}). Normalised muscle power output was calculated as power output divided by wet muscle mass (W kg^{-1}). Each muscle was dried for 48 h in an oven at 37°C, moved to room temperature for 1 h and then reweighed to calculate dry muscle mass (to the nearest 0.0001 g) and water content (% wet mass).

Statistical analysis

Frog length (mm) was not correlated with any of the parameters recorded (Pearson correlation; $P>0.05$), therefore data sets were not normalised to body size (*SVL*) prior to analysis. Dispersion measurements are given as standard error. In all cases $P=0.05$ was accepted for statistical significance.

Muscle morphometrics

Frog sizes and fibre size distributions were compared using a rank sum *t*-test, while muscle morphological data was analysed by two-way ANOVA using SigmaStat™. Where differences were detected they were localised by a Holm–Sidak multiple comparison test.

Muscle mechanics

In most cases control and experimental results were compared using independent sample *t*-tests with correction for unequal variances where appropriate. Control to treatment comparison of power output-cycle frequency data was performed using ANCOVA, with treatment as the factor and cycle frequency as the covariate. Control to treatment comparison of relative power output data during the fatigue tests was performed using ANCOVA, with treatment as the factor and cumulative power as the covariate.

Results

Gross morphology

There was no significant difference in dry muscle mass between either muscle type ($F_{1,20}=0.616$, $P=0.443$) or treatment group ($F_{1,20}=0.939$, $P=0.346$) (Fig. 1A). There was no overall difference in muscle cross-sectional area (CSA) between control and 9-month aestivator muscles ($F_{1,21}=0.762$, $P=0.394$), though there were significant differences between muscle types, with the sartorius muscle having a significantly smaller CSA compared to the iliofibularis ($F_{1,21}=13.102$, $P=0.002$) (Fig. 1B).

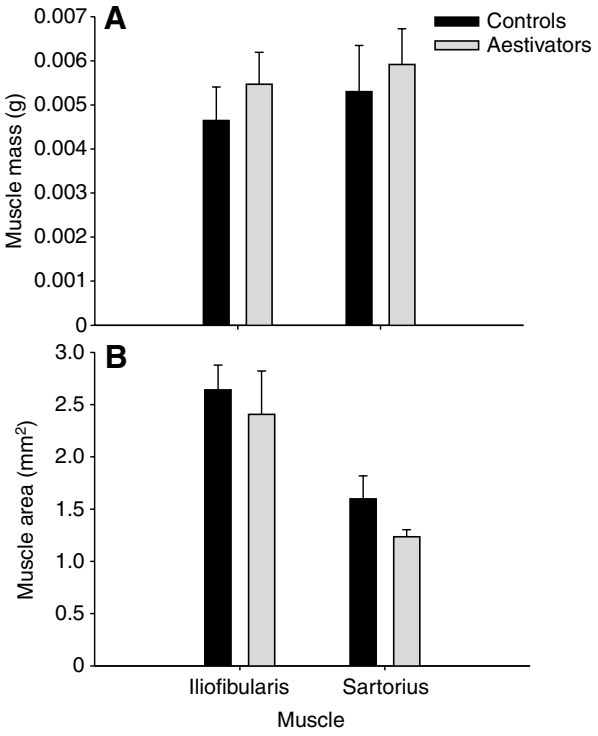

Fig. 1. Effect of aestivation on iliofibularis and sartorius muscle mass and cross-sectional area in the green-striped burrowing frog *Cyclorana alboguttata*. (A) Dry muscle mass (g) of control (N=6) and 9-month aestivator (N=5) iliofibularis and control (N=5) and 9-month aestivator (N=5) sartorius muscles. (B) Whole muscle cross-sectional area (mm^2) of control and 9-month aestivator iliofibularis and sartorius muscles (N=5 for each group). Values are means ± s.e.m.

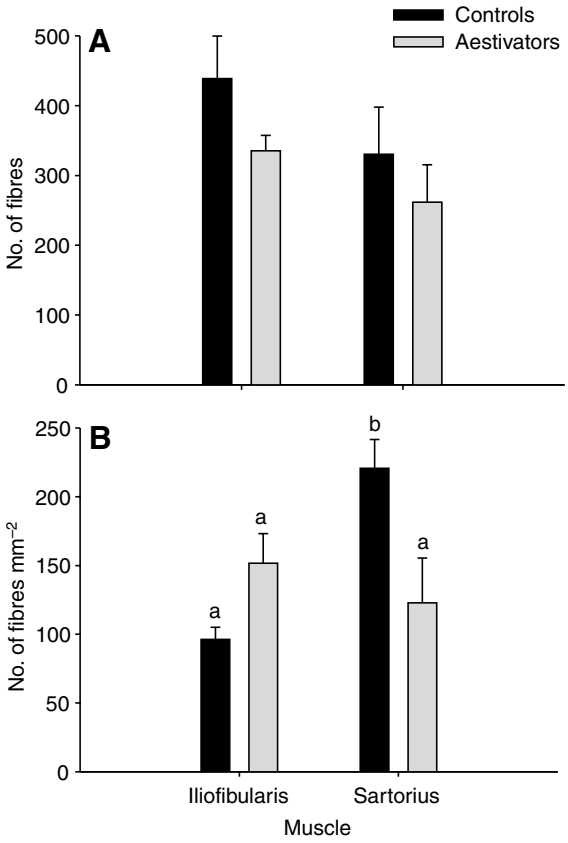

Fig. 2. Effect of aestivation on skeletal muscle fibre number and density of the iliofibularis and sartorius muscles from the green-striped burrowing frog *Cyclorana alboguttata*. (A) Total number of fibres within the iliofibularis and sartorius muscles of control and 9-month aestivator frogs (N=5 for both groups). (B) Density of fibres mm^{-2} within the iliofibularis and sartorius muscles of control and 9-month aestivator frogs (N=5 for both groups). The letters a and b indicate significantly different data sets (P<0.05). Values are means ± s.e.m.

Muscle morphology

There was no significant difference in the total number of fibres present in the muscle cross-sections, between either muscle type ($F_{1,19}$=2.863, P=0.110) or treatment group ($F_{1,19}$=2.557, P=0.129) (Fig. 2A). Overall, there was no significant difference in fibre density between muscle type ($F_{1,19}$=4.485, P=0.06) or treatment group ($F_{1,19}$=0.882, P=0.362) (Fig. 2B), though there was a significant interaction between muscle type and treatment group ($F_{1,19}$=11.53, P=0.004). A Holm–Sidak multiple comparison test revealed that control sartorius fibre density was significantly greater than 9-month aestivator sartorius fibre density (P=0.007) and control iliofibularis density (P=0.001). Control sartorius density was not more dense than 9-month aestivator iliofibularis density, but only arbitrarily (P=0.050). This suggests that, overall, sartorius muscle fibres were more densely packed within the muscle. There was no significant difference in the relative proportions of muscle fibre types (oxidative *versus* glycolytic) between muscle type ($F_{1,7}$=0.000175, P=0.992) or treatment group ($F_{1,7}$=0.000175, P=0.992) (Table 1). Oxidative fibres accounted for greater than 63% of the total number of fibres within each muscle, with the control iliofibularis possessing the greatest proportion of oxidative fibres (76%). Glycolytic fibres accounted for approximately 24% of the total number of fibres within each muscle, the greatest proportion of which was within the 9-month treatment sartorius muscle (37%).

Fibre morphology

There was a significant difference in the CSA of individual fibres between fibre type ($F_{1,41}$=48.54, P<0.001) and treatment group ($F_{3,41}$=17.15, P<0.001). Within the iliofibularis muscle, the CSA of oxidative fibres from 9-month aestivators were significantly smaller than the control oxidative fibres (P=0.041), and 9-month aestivator glycolytic fibres were significantly smaller than control glycolytic fibres (P<0.001) (Fig. 3A). There was no significant difference between treatment groups for fibre CSA of the sartorius muscle (P=0.221), though there was a significant difference between fibre types: glycolytic fibres were significantly larger than oxidative fibres in both controls (P=0.002) and 9-month aestivators (P=0.037) (Fig. 3B). There was no significant difference in the relative size distribution of fibres between

Table 1. *Proportion of fibre types (oxidative versus glycolytic) within the iliofibularis and sartorius muscles of control and 9-month aestivator Cyclorana alboguttata*

Muscle fibre types (%)	Control	Aestivator	*P*
Iliofibularis			
Oxidative	75.8±4.1	65.9±1.6	0.10
Glycolytic	24.2±4.1	34.1±1.6	0.10
Sartorius			
Oxidative	67.4±2.8	63.3±2.3	0.42
Glycolytic	32.4±2.8	36.7±2.3	0.40

N=5 for both groups.

treatment group or muscle type ($P>0.05$), except for the iliofibularis muscle. In this case, 9-month aestivator oxidative fibres were skewed left, with significantly more fibres occurring in the smallest size classes ($P=0.045$) (Fig. 4A).

Isometric properties

Twitch activation, twitch relaxation and tetanus relaxation times were significantly longer in the 9-month aestivator iliofibularis muscle than in the control iliofibularis muscle (Table 2; $P<0.01$); however there were no significant differences in the sartorius muscle. There was no significant difference in tetanus activation time between control and 9-month aestivators for either the iliofibularis or sartorius muscles. Maximum twitch stress and maximum tetanic stress tended to increase with aestivation in both the iliofibularis and sartorius muscles (Table 2; $P>0.05$).

Work loop performance

Nine-months aestivation did not affect the peak power output produced by either the iliofibularis or sartorius muscles (Table 2). Power output-cycle frequency relationships did not differ between control and treatment iliofibularis muscles (Fig. 5A; $P=0.29$). Aestivation caused a significant shift in the sartorius muscle power output-cycle frequency curve (Fig. 5B; $P=0.004$), with treatment muscles producing lower power output at higher cycle frequencies. Control sartorius muscles produced higher maximal power output at a higher cycle frequency than the iliofibularis muscle, with the 9-month aestivator sartorius muscle producing a power output-cycle frequency curve similar to that of the iliofibularis. Aestivation tended to reduce fatigue resistance in both the iliofibularis and the sartorius muscles, as evidenced by a more rapid initial decline in 9-month aestivator muscle power output during bouts of repeated work loop cycles (Fig. 6A,B; $P=0.53$ and $P=0.09$, respectively).

Discussion

The results of this study clearly demonstrate that hindlimb muscles of *Cyclorana alboguttata* do not exhibit a typical atrophic response to prolonged immobilisation during aestivation. Prolonged aestivation of 9-months duration had little or no effect on either the morphology or contractile properties of the iliofibularis and sartorius muscles.

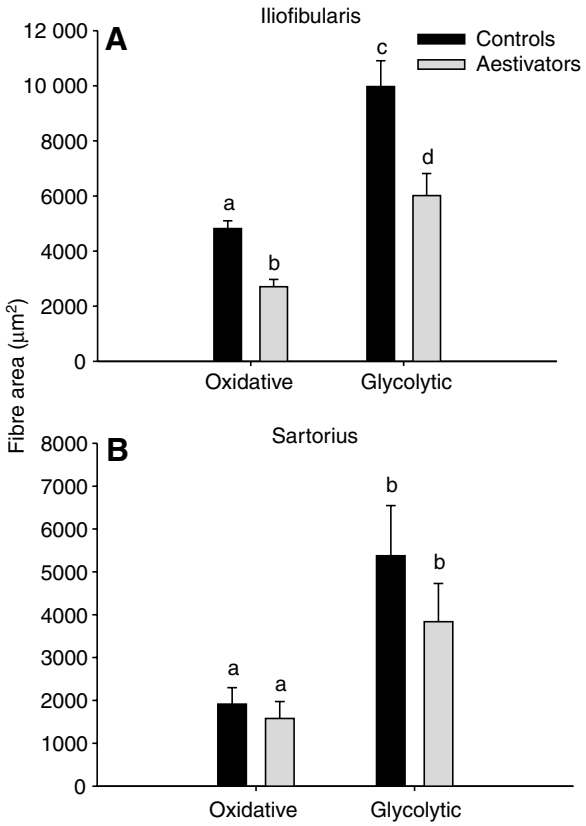

Fig. 3. Fibre cross-sectional areas (μm^2) of (A) the iliofibularis and (B) sartorius muscles for control and aestivator groups from the Green-striped burrowing frog, *Cyclorana alboguttata* (*N*=5 for both groups). Letters a–d indicate significantly different data sets ($P<0.05$). Values are means ± s.e.m.

Muscle morphology

The sartorius and iliofibularis muscles of the 9-month aestivators maintained dry muscle mass and whole muscle cross-sectional area at control levels (Fig. 1A,B). Both sartorius and iliofibularis act to abduct the femur and flex the knee (Duellman and Trueb, 1994), therefore these are not the main extensor muscles used to power jumping. As such, they might represent skeletal muscles less likely to be protected from atrophy during a challenge such as aestivation.

The absence of change at the whole muscle level is in stark contrast to the effects of immobilisation or dormancy previously reported in other animals (Adams et al., 2003; Baldwin and Haddad, 2001; Booth, 1977; Booth and Seider, 1979b). Similar significant reductions in muscle mass were observed in guinea pig muscle after immobilisation for 28 days (Maier et al., 1976). Hibernation caused significant atrophy in the muscles of golden-mantled ground squirrels *Spermophilus lateralis* (Wickler et al., 1991a), big brown bats *Eptesiscus fuscus* (Yacoe, 1983) and hamsters (Wickler et al., 1987). However, aestivation for 3 and 6 months in *C. alboguttata* has

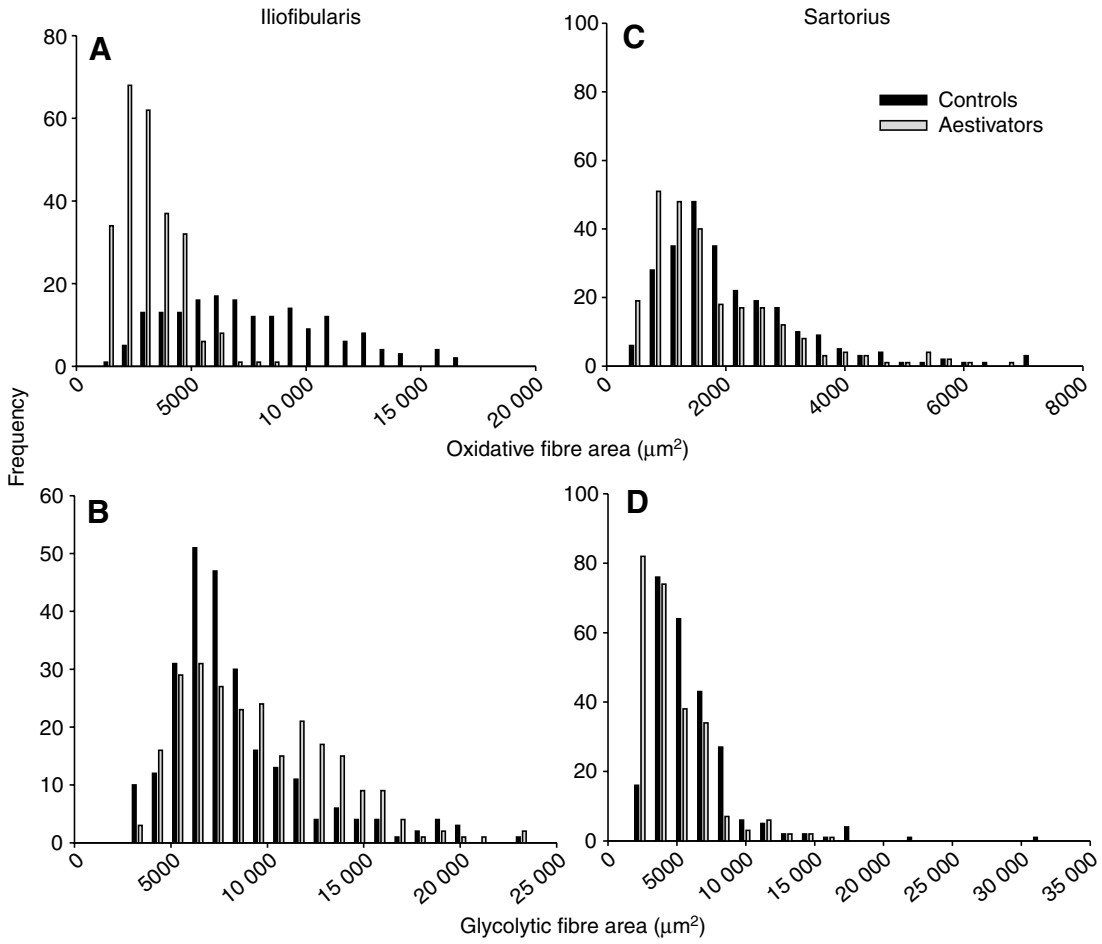

Fig. 4. Effect of aestivation on size frequency distribution of control and 9-month aestivator iliofibularis muscle (A) oxidative fibres and (B) glycolytic fibres (N=5 for both groups), and control and 9-month aestivator sartorius muscle (C) oxidative and (D) glycolytic fibres (N=5 for both groups). Fibre size is expressed as cross-sectional area in μm^2. Values are means ± s.e.m.

previously been shown to have no effect on the wet muscle masses of locomotory muscles (Hudson and Franklin, 2002a; Hudson et al., 2006).

There were no significant changes to total fibre number between control and 9-month aestivator groups for either muscle type in *C. alboguttata* (Fig. 2A), which rules out loss of fibres (hypoplasia) during aestivation. Fibre density declined significantly after aestivation in the sartorius muscle only (Fig. 2B). Combined with the maintenance of sartorius dry muscle mass and fibre number, it is possible that this reduction in fibre density reflects an increase in sartorius muscle length during aestivation. Aestivating *C. alboguttata* adopt a characteristic water-conserving posture (Withers and Richards, 1995), and it is possible that this behaviour might confer a postural advantage to selected hindlimb muscles, such as the sartorius, by maintaining the muscle in a lengthened position. It has been demonstrated in previous studies that fixing muscle in the lengthened position during immobilisation can delay the onset of muscle disuse atrophy (Booth, 1977; Goldspink, 1977). Our results are unlike previous studies in which the cross-sectional areas of muscle fibres and numbers of fibres have significantly decreased after immobilisation and dormancy. Immobilisation caused significant reductions in the diameter of guinea pig soleus muscle fibres after 21 days of immobilisation (Tomanek and Lund, 1974), the cross-sectional area of cat tibialis muscle (Nordstrom et al., 1995) and fibre number in rat soleus muscle (Booth and Kelso, 1973). Hibernation caused a significant reduction in the cross-sectional areas of fibres from the soleus and extensor digitorum longus muscles of hibernating golden-mantled ground squirrels *Spermophilus lateralis* (Steffen et al., 1991) though not in hibernating black bears *Ursus americanus* (Tinker et al., 1998).

The most dramatic morphological change observed in this study was the significant reduction in iliofibularis muscle fibre cross-sectional area (Fig. 3A). Based on the results of previous studies (e.g. Booth and Kelso, 1973; Maier et al., 1976; Tomanek and Lund, 1974) we had predicted that the predominantly oxidative iliofibularis muscle would be more susceptible to muscle disuse atrophy than the glycolytic sartorius muscle. During normal activity, oxidative muscles tend to be used frequently, for extended periods of time and at low intensities. In comparison, fast-twitch glycolytic muscles

Table 2. *Effect of aestivation on skeletal muscle work loop power output, isometric kinetics and maximal isometric stress*

	Iliofibularis			Sartorius		
	Control	Aaestivator	*P*	Control	Aaestivator	*P*
Time (ms)						
To peak twitch	33.9±1.8	45.0±1.6	0.003	33.6±1.3	38.2±2.1	0.09
From peak twitch to half relaxation	21.9±1.9	33.0±0.7	0.001	24.2±2.6	35.3±4.6	0.07
To half peak tetanus	38.5±2.5	39.4±1.7	0.81	25.9±1.3	25.2±0.3	0.60
From last stimulus to half tetanus relaxation	71.1±5.8	89.3±1.4	0.02	73.3±3.8	83.3±4.1	0.11
Maximum stress (kN m^{-2})						
Twitch	119±10	191±30	0.09	104±14	145±16	0.09
Tetanic stress	484±38	611±55	0.08	258±31	301±30	0.34
Peak power output (W kg^{-1})	61.3±4.5	59.3±2.5	0.70	66.9±4.0	60.9±4.3	0.34

Values are means ± s.e.m. (*N*=6 for controls and *N*=5 for aestivators). *P* values given are for *t*-tests.

are used infrequently, for short periods and at high intensity. When oxidative muscles become inactive the scope of the change in the level of activity is much greater than that experienced by glycolytic, fast-twitch muscles (Hudson and Franklin, 2002b). Additionally, due to the comparatively greater numbers of mitochondria contained within them, oxidative fibres produce comparatively larger quantities of reactive oxygen species as a byproduct of normal cell metabolism. Reactive oxygen species, or free radicals, are responsible for stochastically damaging cell architecture, including proteins and lipids, which leads to cellular apoptosis and hence muscle disuse atrophy (Kondo et al., 1993a; Kondo et al., 1993b; Kondo et al., 1993c; Kondo et al., 1994). The dramatic downregulation of activity in the oxidative muscle may trigger profound changes in the biochemical function of the muscle mitochondria. As such, it is possible that the susceptibility of oxidative muscles to muscle disuse atrophy is due to changes in mitochondrial density or architecture rather than compositional fibre changes. In other words, the ultimate consequences of muscle disuse atrophy may be more important at the whole muscle level rather than at the individual fibre level.

The reduction in cross-sectional area of the iliofibularis muscle fibres, but not sartorius muscle fibres, supports our initial hypothesis; however, the results of the histochemical analysis show that the iliofibularis muscle of *C. alboguttata* is not a homogenously oxidative muscle (Table 1). If oxidative fibres are more susceptible to muscle disuse atrophy than glycolytic fibres, we would anticipate that the oxidative fibres would have been preferentially affected over the glycolytic fibres. In actuality, the significant reduction in fibre cross-sectional area in the iliofibularis muscle of 9-month aestivators occurred in both oxidative and glycolytic fibre types (Fig. 3A).

The enzyme histochemistry component of this study does not support the hypothesis that prolonged aestivation induces the transition of fibre types from characteristically (histologically) slow, oxidative to fast, glycolytic. The relative proportions of the two fibre types, measured as a percentage of total number of fibres present in the muscle cross-section, was consistent between controls and 9-month aestivators (Table 1). Furthermore, the size-distribution of fibres, as measured by cross-sectional area, remained consistent between controls and 9-month aestivators (Fig. 4A–D). There was a trend for larger iliofibularis glycolytic fibres (>10 000 μm^2) to occur more frequently after 9 months of aestivation; however, it was not significant (Fig. 4B).

Muscle mechanics

Previous research has demonstrated that prolonged aestivation has no effect on skeletal motor nerve terminals or membrane potentials in *C. alboguttata* (Hudson et al., 2005); however, the effect on muscle mechanics was more complex. Twitch activation, twitch relaxation and tetanus relaxation times were significantly longer in 9-month aestivator iliofibularis muscle, but there were no significant changes in sartorius muscle (Table 2). The findings for the faster twitch sartorius muscle concur with previous work on 3-month aestivator *C. alboguttata* gastrocnemius muscle (Hudson and Franklin, 2002a), suggesting that the isometric kinetics of relatively fast muscles are unaffected by aestivation. The apparent slowing of isometric kinetics of the relatively slower twitch iliofibularis muscle was contrary to the findings of most previous studies (Maier et al., 1973; Nordstrom et al., 1995) and the reverse of our hypothesis. However, the slowing of isometric kinetics in our study was consistent with other findings (Rourke, 2004), which showed a non-significant tendency for the expression of myosin heavy chain protein isoforms to shift towards slower isoforms in hibernating ground squirrels. The observed fast-to-slow transition in squirrels may be due to shivering thermogenesis during periodic arousal from hibernation; however, this is clearly not the case for an amphibian aestivator.

There were no significant differences in isometric stress between controls and 9-month aestivators for either sartorius or iliofibularis muscles (Table 2). This is similar to the results found previously in 3-month aestivator *C. alboguttata* gastrocnemius muscle (Hudson and Franklin, 2002a). 12–16 weeks of aquatic hibernation in *Rana temporaria* has also been previously shown not to affect sartorius muscle isometric stress (West et al., 2006), therefore skeletal muscle stress appears to

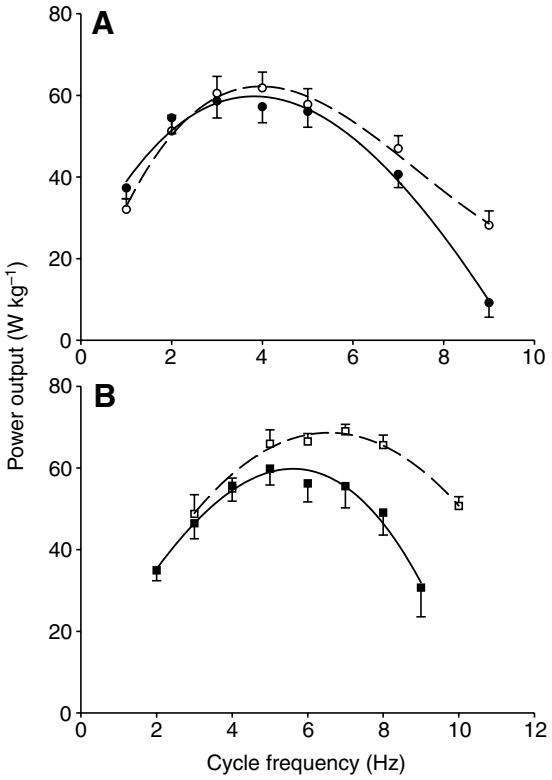

Fig. 5. Effect of aestivation on skeletal muscle power output-cycle frequency relationships determined *via* the work-loop technique for (A) iliofibularis and (B) sartorius muscles. Open symbols, control aestivator groups; closed symbols, 9-month aestivator groups (N=6 and N=5, respectively). Values are means ± s.e.m. The broken and solid lines represent cubic polynomial functions fitted to the control and treatment data, respectively.

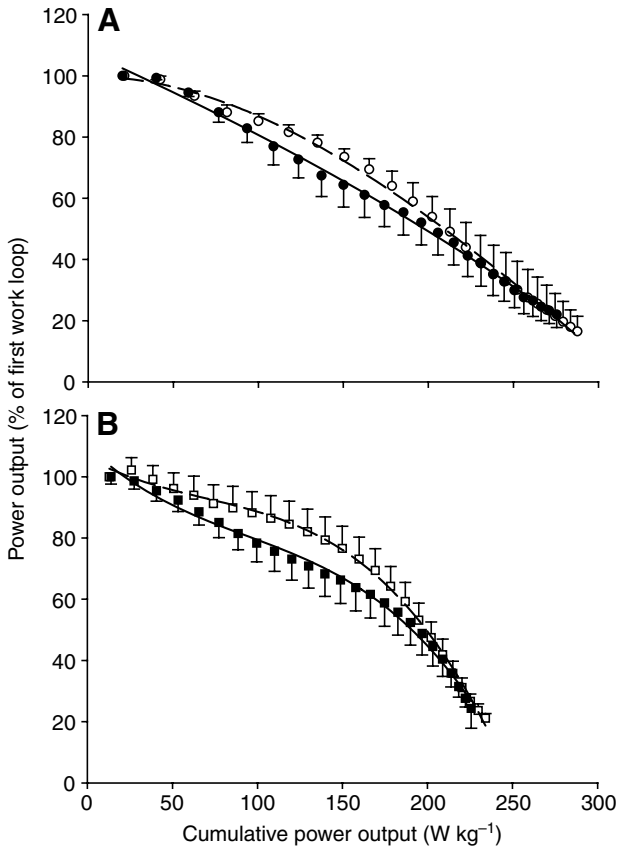

Fig. 6. Effect of aestivation on skeletal muscle fatigue resistance during a series of work loops for (A) iliofibularis and (B) sartorius muscles. Open symbols, control aestivator groups; closed symbols, 9-month aestivator groups (N=6 and N=5, respectively). Values are means ± s.e.m. The broken and solid lines represent cubic polynomial functions fitted to the control and treatment data, respectively.

be conserved in the natural models of anuran dormancy studied so far.

There were no aestivation-related changes to power output-cycle frequency curves in the slow-twitch iliofibularis of *C. alboguttata*, but the fast-twitch sartorius became slower after 9 months of aestivation (Fig. 5B), which is contrary to the results obtained from previous studies (Diffee et al., 1991) and the reverse of our hypothesis. This result may have been indicative of fibre-type remodelling of the sartorius, though there was no evidence to support this (Table 1). In contrast, sartorius muscle isovelocity power output remained stable after 12–16 weeks of aquatic hibernation in *Rana temporaria*, suggesting that the muscle mechanical properties of over-wintering frogs remain stable. 9 months of aestivation had no significant effect on fatigue resistance in either the iliofibularis or sartorius muscles (Fig. 6A,B), which was similar to the findings in the gastrocnemius muscle from 3-month aestivator *C. alboguttata* (Hudson and Franklin, 2002a).

Within the present study, the effect of aestivation on isometric kinetics of skeletal muscle partially contradicts the findings from work-loop assessment of mechanical properties. However, previous studies have demonstrated that isometric kinetics and force–velocity properties alone are poor predictors of work-loop shape (Caiozzo, 2002; James et al., 1996). Work-loop shape is also affected by force enhancement, shortening deactivation and passive properties of muscle (Josephson, 1993). Therefore, the work-loop technique can give a more functionally relevant estimate of muscular performance capabilities by assessing performance under dynamic conditions (Josephson, 1993).

The apparent slowing of both muscle types, as evidenced by the isometric contractile properties data, indicated that prolonged aestivation did not induce a transition from slow to fast twitch in the iliofibularis and sartorius muscles. As such, prolonged aestivation may have an effect on muscle physiology or biochemistry that indirectly changes the isometric properties of the muscle tissue without affecting fatigue resistance. Changes to muscle mitochondrial structure, function or density may be one possible explanation, though it has been demonstrated that mitochondrial density is not correlated with contraction speed and fatigability in individual fibres (Lipska et al., 1998). A decline in the energetic stores of the individual muscles, rather than fibre type changes, may be responsible for

this outcome; however, levels of muscle metabolites, such as phosphorylated creatine and ATP, are known to remain stable in the muscles of cold-submerged hibernating *R. temporaria* (West et al., 2006), though it should be noted that the quiescent period of this anuran species is substantially shorter than that experienced by *C. alboguttata*. Furthermore, it has been postulated that one of the mechanisms acting to protect animals from muscle disuse atrophy during dormancy is low body temperature, in conjunction with lowered metabolic rate (Hudson and Franklin, 2002b). In this way, *C. alboguttata* is truly remarkable as it is the only animal model ever investigated that does not exhibit any muscle disuse atrophy during a temperature-independent dormancy.

Concluding remarks

The present study demonstrated that 9 months of aestivation had little effect on muscle morphology, histochemistry and dynamic mechanical performance in *C. alboguttata*. There was no skeletal muscle atrophy of the type normally associated with either experimental limb disuse or hibernation. Whole muscle mass, muscle cross-sectional area, fibre number and proportions of fibre types remained unchanged after prolonged disuse, and relatively minor morphological changes to fibre cross-sectional area and density were observed.

Overall, prolonged aestivation had little effect on the isometric properties of the skeletal muscle of *C. alboguttata*, with moderate, though significant, changes occurring in the slow-twitch iliofibularis muscle. Though the results of the isometric analysis suggested that the muscles were becoming slower after 9 months of aestivation, the results of the work-loop analysis clearly demonstrate that the contractile properties of aestivating *C. alboguttata* are maintained at control levels. Despite the fact that significant changes are occurring at the muscle structural level, overall muscle performance, and hence whole animal performance, is preserved. Further investigation is required to elucidate the mechanisms that prevent skeletal muscle disuse atrophy during aestivation.

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References

Adams, G. R., Caiozzo, V. J. and Baldwin, K. M. (2003). Skeletal muscle unweighting: spaceflight and ground-based models. *J. Appl. Physiol.* **95**, 2185-2201.

Anderson, J., Almeida-Silveira, M. I. and Perot, C. (1999). Reflex and muscular adaptations in rat soleus muscle after hindlimb suspension. *J. Exp. Biol.* **202**, 2701-2707.

Ariano, M. A., Armstrong, R. B. and Edgerton, V. R. (1973). Hindlimb muscle fiber populations of 5 mammals. *J. Histochem. Cytochem.* **21**, 51-55.

Baldwin, K. M. and Haddad, F. (2001). Plasticity in skeletal, cardiac and smooth muscle. Invited review: effects of different activity and inactivity paradigms on myosin heavy chain gene expression in striated muscle. *J. Appl. Physiol.* **90**, 345-357.

Booth, F. W. (1977). Time course of muscular atrophy during immobilization of hindlimbs in rats. *J. Appl. Physiol.* **43**, 656-661.

Booth, F. W. (1982). Effect of limb immobilization on skeletal muscle. *J. Appl. Physiol.* **52**, 1113-1118.

Booth, F. W. and Kelso, J. R. (1973). Effect of hind limb immobilization on contractile and histochemical properties of skeletal muscle. *Pflugers Arch.* **342**, 231-238.

Booth, F. W. and Seider, M. J. (1979a). Early change in skeletal muscle protein synthesis after limb immobilization of rats. *J. Appl. Physiol.* **47**, 974-977.

Booth, F. W. and Seider, M. J. (1979b). Recovery of skeletal muscle after 3 months of hind limb immobilization in rats. *J. Appl. Physiol.* **47**, 435-439.

Caiozzo, V. J. (2002). Plasticity of skeletal muscle phenotype: mechanical consequences. *Muscle Nerve* **26**, 740-768.

Canon, F. and Goubel, F. (1995). Changes in stiffness induced by hindlimb suspension in rat soleus muscle. *Pflugers Arch.* **429**, 332-337.

Carey, H. V., Andrews, M. T. and Martin, S. L. (2003). Mammalian hibernation: cellular and molecular responses to depressed metabolism and low temperature. *Physiol. Rev.* **83**, 1153-1181.

Cramp, R. L. and Franklin, C. E. (2005). Arousal and re-feeding rapidly restores digestive tract morphology following aestivation in green-striped burrowing frogs. *Comp. Biochem. Physiol.* **142A**, 451-460.

Diffee, G. M., Caiozzo, V. J., Herrick, R. E. and Baldwin, K. M. (1991). Contractile and biochemical properties of rat soleus and plantaris after hindlimb suspension. *Am. J. Physiol.* **260**, C528-C534.

Duellman, W. E. and Trueb, L. (1994). *Biology of Amphibians*. New York: McGraw-Hill.

Edgerton, V. R., Roy, R. R., Allen, D. L. and Monti, R. J. (2002). Adaptations in skeletal muscle disuse or decreased-use atrophy. *Am. J. Phys. Med. Rehabil.* **81**, S127-S147.

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

Frerichs, K. U., Smith, C. B., Brenner, M., DeGracia, D. J., Krause, G. S., Marrone, L., Dever, T. E. and Hallenbeck, J. M. (1998). Suppression of protein synthesis in brain during hibernation involves inhibition of protein initiation and elongation. *Proc. Natl. Acad. Sci. USA* **95**, 14511-14516.

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

Goldspink, D. F. (1977). Influence of immobilization and stretch on protein turnover of rat skeletal-muscle. *J. Physiol. Lond.* **264**, 267-282.

Guppy, M. and Withers, P. (1999). Metabolic depression in animals: physiological perspectives and biochemical generalizations. *Biol. Rev.* **74**, 1-40.

Harlow, H. J., Lohuis, T., Beck, T. D. I. and Iaizzo, P. A. (2001). Muscle strength in overwintering bears – unlike humans, bears retain their muscle tone when moribund for long periods. *Nature* **409**, 997.

Hudson, N. J. and Franklin, C. E. (2002a). Effect of aestivation on muscle characteristics and locomotor performance in the Green-striped burrowing frog, *Cyclorana alboguttata*. *J. Comp. Physiol. B* **172**, 177-182.

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

Hudson, N. J. and Franklin, C. E. (2003). Preservation of three-dimensional capillary structure in frog muscle during aestivation. *J. Anat.* **202**, 471-474.

Hudson, N. J., Bennett, M. B. and Franklin, C. E. (2004). Effect of aestivation on long bone mechanical properties in the green-striped burrowing frog, *Cyclorana alboguttata*. *J. Exp. Biol.* **207**, 475-482.

Hudson, N. J., Lavidis, N. A., Choy, P. T. and Franklin, C. E. (2005). Effect of prolonged inactivity on skeletal motor nerve terminals during aestivation in the burrowing frog, *Cyclorana alboguttata*. *J. Comp. Physiol. A* **191**, 373-379.

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

James, R. S., Young, I. S., Cox, V. M., Goldspink, D. F. and Altringham, J. D. (1996). Isometric and isotonic muscle properties as determinants of work loop power output. *Pflugers Arch.* **432**, 767-774.

Josephson, R. K. (1993). Contraction dynamics and power output of skeletal muscle. *Annu. Rev. Physiol.* **55**, 527-546.

Kandarian, S. C. and Stevenson, E. J. (2002). Molecular events in skeletal muscle during disuse atrophy. *Exerc. Sport Sci. Rev.* **30**, 111-116.

Kobelt, F. and Linsenmair, K. E. (1995). Adaptations of the reed frog *Hyperolius viridiflavus* (Amphibia, Anura, Hyperoliidae) to its arid environment. VII. The heat budget of *Hyperolius viridiflavus nitidulus* and the evolution of an optimized body shape. *J. Comp. Physiol. B* **165**, 110-124.

Kondo, H., Kodama, J., Kishibe, T. and Itokawa, Y. (1993a). Oxidative stress during recovery from muscle atrophy. *FEBS Lett.* **326**, 189-191.

Kondo, H., Miura, M. and Itokawa, Y. (1993b). Antioxidant enzyme systems in skeletal muscle atrophied by immobilization. *Pflugers Arch.* **422**, 404-406.

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

Kondo, H., Nishino, K. and Itokawa, Y. (1994). Hydroxyl radical generation in skeletal muscle atrophied by immobilization. *FEBS Lett.* **349**, 169-172.

Lipska, E., Novotova, M., Radzyukevich, T. and Nasledov, G. (1998). Is there an explicit correspondence between physiological and morphological features in amphibian twitch skeletal muscle fibres? *Basic Appl. Myol.* **8**, 405-412.

Maier, A., Eldred, E. and Edgerton, V. R. (1973). The effects on spindles of muscle atrophy and hypertrophy. *Exp. Neurol.* **37**, 100-123.

Maier, A., Crockett, J. L., Simpson, D. R., Saubert, C. W. and Edgerton, V. R. (1976). Properties of immobilized guinea-pig hindlimb muscles. *Am. J. Physiol.* **231**, 1520-1526.

Mendez, J. and Keys, A. (1960). Density and composition of mammalian muscle. *Metabolism* **9**, 184-189.

Meyer, E., Jamieson, B. G. M. and Scheltinga, D. M. (1997). Sperm ultrastructure of six Australian hylid frogs from two genera (*Litoria* and *Cyclorana*): phylogenetic implications. *J. Submicrosc. Cytol. Pathol.* **29**, 443-451.

Musacchia, X. J., Steffen, J. M. and Fell, R. D. (1988). Disuse atrophy of skeletal muscle: animal models. *Exerc. Sport Sci. Rev.* **16**, 61-87.

Nordstrom, M. A., Enoka, R. M., Reinking, R. M., Callister, R. C. and Stuart, D. G. (1995). Reduced motor unit activation of muscle spindles and tendon organs in the immobilized cat hindlimb. *J. Appl. Physiol.* **78**, 901-913.

Pakay, J. L., Withers, P. C., Hobbs, A. A. and Guppy, M. (2002). In vivo downregulation of protein synthesis in the snail *Helix apersa* during estivation. *Am. J. Physiol.* **283**, R197-R204.

Pedler, S., Fuery, C. J., Withers, P. C., Flanigan, J. and Guppy, M. (1996). Effectors of metabolic depression in an estivating pulmonate snail (*Helix aspersa*): Whole animal and in vitro tissue studies. *J. Comp. Physiol. B* **166**, 375-381.

Peter, J. B., Barnard, R. J., Edgerton, V. R., Gillespi, C. A. and Stempel, K. E. (1972). Metabolic profiles of three fiber types of skeletal muscle in guinea pigs and rabbits. *Biochemistry* **11**, 2627-2633.

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

Putnam, R. W. and Bennett, A. F. (1983). Histochemical, enzymatic, and contractile properties of skeletal muscles of three anuran amphibians. *Am. J. Physiol.* **244**, R558-R567.

Rourke, B. C., Yokoyama, Y., Milsom, W. K. and Caiozzo, V. J. (2004). Myosin isoform expression and MAFbx mRNA levels in hibernating golden-mantled ground squirrels (*Spermophilus lateralis*). *Physiol. Biochem. Zool.* **77**, 582-593.

Rowlerson, A. M. and Spurway, N. C. (1988). Histochemical and immunohistochemical properties of skeletal muscle fibers from *Rana* and *Xenopus*. *Histochem. J.* **20**, 657-673.

Roy, R. R., Baldwin, K. M. and Edgerton, V. R. (1991). The plasticity of skeletal muscle: effects of neuromuscular activity. *Exerc. Sport Sci. Rev.* **19**, 269-312.

Roy, R. R., Eldridge, L., Baldwin, K. M. and Edgerton, V. R. (1996). Neural influence on slow muscle properties: Inactivity with and without cross-reinnervation. *Muscle Nerve* **19**, 707-714.

Roy, R. R., Zhong, H., Hodgson, J. A., Grossman, E. J., Siengthai, B., Talmadge, R. J. and Edgerton, V. R. (2002). Influences of electromechanical events in defining skeletal muscle properties. *Muscle Nerve* **26**, 238-251.

Seymour, R. S. (1973). Energy metabolism of dormant spadefoot toads (*Scaphiopus*). *Copeia* **1973**, 435-445.

Steffen, J. M., Koebel, D. A., Musacchia, X. J. and Milsom, W. K. (1991). Morphometric and metabolic indexes of disuse in muscles of hibernating ground-squirrels. *Comp. Biochem. Physiol.* **99B**, 815-819.

Tinker, D. B., Harlow, H. J. and Beck, T. D. I. (1998). Protein use and muscle-fiber changes in free-ranging, hibernating black bears. *Physiol. Zool.* **71**, 414-424.

Tomanek, R. J. and Lund, D. D. (1974). Degeneration of different types of skeletal muscle fibers. 2. Immobilization. *J. Anat.* **118**, 531-541.

Trappe, S., Trappe, T., Gallagher, P., Harber, M., Alkner, B. and Tesch, P. (2004). Human single muscle fibre function with 84 day bed-rest and resistance exercise. *J. Physiol. Lond.* **557**, 501-513.

van Beurden, E. K. (1980). Energy metabolism of dormant Australian Water-holding frogs (*Cyclorana platycephala*). *Copeia* **1980**, 787-799.

West, T. G., Donohoe, P. H., Staples, J. F. and Askew, G. N. (2006). Tribute to R. G. Boutilier: the role for skeletal muscle in the hypoxia-induced hypometabolic responses of submerged frogs. *J. Exp. Biol.* **209**, 1159-1168.

Wickler, S. J., Horwitz, B. A. and Kott, K. S. (1987). Muscle function in hibernating hamsters – a natural analog to bed rest. *J. Therm. Biol.* **12**, 163-166.

Wickler, S. J., Hoyt, D. F. and Van Breukelen, F. (1991a). Disuse atrophy in the hibernating golden-mantled ground squirrel, *Spermophilus lateralis*. *Am. J. Physiol.* **261**, 1214-1217.

Wickler, S. J., Hoyt, D. F. and Vanbreukelen, F. (1991b). Disuse atrophy in the hibernating golden-mantled ground squirrel, *Spermophilus lateralis*. *Am. J. Physiol.* **261**, R1214-R1217.

Withers, P. C. (1995). Cocoon formation and structure in the estivating Australian desert frogs, *Neobatrachus* and *Cyclorana*. *Aust. J. Zool.* **43**, 429-441.

Withers, P. C. (1998). Evaporative water loss and the role of cocoon formation in Australian frogs. *Aust. J. Zool.* **46**, 405-418.

Withers, P. C. and Richards, S. J. (1995). Cocoon formation by the tree frog *Litoria alboguttata* (Amphibia: Hylodae). A waterproof taxonomic tool. *J. R. Soc. West. Aust.* **78**, 103-106.

Witzmann, F. A., Kim, D. H. and Fitts, R. H. (1982). Recovery time course in contractile function of fast and slow skeletal muscle after hindlimb immobilization. *J. Appl. Physiol.* **52**, 677-682.

Yacoe, M. E. (1983). Maintenance of the pectoralis muscle during hibernation in the big brown bat, *Eptesicus fuscus*. *J. Comp. Physiol.* **152**, 97-104.