Lessons from an estivating frog: sparing muscle protein despite starvation and disuse

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Am J Physiol Regul Integr Comp Physiol 290: R836–R843, 2006. First published October 20, 2005; doi:10.1152/ajpregu.00380.2005.—Long (6- to 9-mo) bouts of estivation in green-striped burrowing frogs lead to 28% atrophy of cruralis oxidative fibers (P < 0.05) and some impairment of in vitro gastrocnemius endurance (P < 0.05) but no significant deficit in maximal twitch force production. These data suggest the preferential atrophy of oxidative fibers at a rate slower than, but comparable to, laboratory disuse models. We tested the hypothesis that the frog limits atrophy by modulating oxidative stress. We assayed various proteins at the transcript level and verified these results for antioxidant enzymes at the biochemical level. Transcript data for NADH ubiquinone oxidoreductase subunit 1 (71% downregulated, P < 0.05) and ATP synthase (67% downregulated, P < 0.05) are consistent with mitochondrial quiescence and reduced oxygen production. Meanwhile, uncoupling protein type 2 transcription (P = 0.31), which is thought to reduce mitochondrial leakage of reactive oxygen species, was maintained. Total antioxidant defense of water-soluble (22.3 ± 1.7 and 23.8 ± 1.5 μM/μg total protein in control and estivator, respectively, P = 0.53) and membrane-bound proteins (31.5 ± 1.9 and 42.1 ± 7.3 μM/μg total protein in control and estivator, respectively, P = 0.18) was maintained, equivalent to a bolstering of defense relative to oxygen insult. This probably decelerates muscle atrophy by preventing accumulation of oxidative damage in static protein reserves. Transcripts of the mitochondrial encoded antioxidant superoxide dismutase type 2 (67% downregulated, P < 0.05) paralleled mitochondrial activity, whereas nuclear-encoded catalase and glutathione peroxidase were maintained at control values (P = 0.42 and P = 0.231), suggesting a dissonance between mitochondrial and nuclear antioxidant expression. Pyruvate dehydrogenase kinase 4 transcription was fourfold lower in estivators (P = 0.11), implying that, in contrast to mammalian hibernators, this enzyme does not drive the combustion of lipids that helps spare hypometabolic muscle.

disuse atrophy; antioxidant; mitochondria; gene expression

THE GREEN-STRIPED BURROWING frog Cyclorana alboguttata survives extended droughts by burrowing underground, shedding a waterproof cocoon (33), and undergoing a deep metabolic depression called estivation (29). In this capacity, its hindlimbs are fully immobilized, and it has no external food supply. C. alboguttata can withstand short (3-mo) bouts of estivation without suffering the losses in muscle contractile performance and mass (13) normally associated with disuse (23) and starvation (12).

Proximally, these losses in mammalian atrophy models are the result of a negative protein balance due to a relative reduction in protein synthesis (30) and a relative increase in protein degradation (6). The ultimate reasons reflect the plastic response of muscle to its contractile history or an energy balance that cannot be met by food and endogenous fat reserves. The ability of C. alboguttata to rapidly emerge from their subterranean burrows suggests that they have certain physiological modifications that limit these effects, thereby permitting opportunistic feeding and breeding when the summer rains come.

Some ability to inhibit muscle atrophy appears to be a relatively common phenomenon in dormant animals, as exemplified by hibernating bears (11), hamsters (32), and squirrels (28), as well as estivating frogs. However, the mechanisms underpinning this phenotype are largely unknown. Metabolic depression per se likely has a protective role (14), in part by delaying the need to mobilize muscle protein. Additionally, in the specific case of C. alboguttata, spontaneous release of the neurotransmitter acetylcholine is maintained at lillofibularis neuromuscular junctions (15), permitting ongoing communication between muscle and nerve, despite an absence of contraction.

In this study, we aimed to further characterize the impact of estivation on muscle structure in extended (6- to 9-mo) bouts of estivation, which more closely approximate the field situation. To this end, we have assayed in vitro muscle performance and muscle fiber composition/morphometry. In addition, we aimed to gain a better understanding of the physiological mechanisms underpinning the inhibition of muscle atrophy by assessing gene expression of several candidate proteins. We used quantitative PCR to test three main hypotheses. First, we examined antioxidant enzymes [glutathione peroxidase 4 (GPX4), catalase (CAT), and superoxide dismutase 2 (SOD2)], because an accumulation of oxidative damage contributes to protein degradation in atrophying mammalian muscle (16, 18–20). We also verified the antioxidant defense transcript data at the biochemical level. Second, we predicted that muscle protein would be spared as a major fuel source. To this end, we tested pyruvate dehydrogenase kinase 4 (PDK4), the biochemical switch indicating a preferential combustion of lipids in hibernating mammals (5). Third, we hypothesized that estivating frogs would increase metabolic efficiency, inasmuch as their food supply is unpredictable and sporadic. To this end, we tested the expression of genes fundamental to mitochondrial energy metabolism and efficiency [uncoupling protein (UCP)]

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Control frogs (treatment groups (controls, 6-mo estivators, and 9-mo estivators), with summer rains in southeastern and mideastern Queensland, Australia, tata burrow line. block. After 6 mo (for cruralis RNA, cruralis histology, and gracilis dry naturally over a period of several days. These frogs burrowed almost immediately and quickly became entombed in a hardened clay block. After 6 mo (for cruralis RNA, cruralis histology, and gracilis biochemistry) or 9 mo (for gastrocnemius contractile properties), we extracted estimating frogs by breaking open the soil block across the burrow line.

The different muscles were selected for various practical reasons. At its distal end, gastrocnemius has a bunch of connective tissue, which provides an excellent site for suturing, making it ideal for in vitro contractile studies. The cruralis is the largest single muscle in the hindlimb; therefore, it was used for RNA extraction, inasmuch as RNA yield can limit the validity of expression analysis. The contralateral cruralis was used for histology to help interpret the gene expression data. The gracilis, which can be identified and excised easily, was chosen for biochemistry. All frogs were euthanized by double pithing the neck desiccation and stored at −80°C, and subsequently used for analysis of membrane-bound proteins.

The antioxidant potential of water-soluble and membrane-bound proteins was assessed using a commercially available colorimetric kit (Total Antioxidant Power; Oxford Biomedical Research) following the manufacturer's instructions. The colorimetric reading was assayed on a multiplate colorimetric spectrophotometer at 490 nm. Total protein content was determined using a commercially available spectrophotometric kit (BSA protein assay; Sigma) assayed at 562 nm. The antioxidant power results are expressed per microgram of total protein.

Muscle mechanics. The gastrocnemius muscle was attached to a force transducer (model FE301; Grass). The gastrocnemius was specified, because it has a bundle of connective tissue that allows convenient suturing. It was tied at the distal end with silk suture material and pinned directly through the knee joint at the proximal end. The muscle was bathed in circulating Ringer solution (pH 7.4) at 24 ± 1°C. The transducer was connected to a MacLab data acquisition system, and the signal was amplified using a Bridge Amp (AD Instruments, Castle Hill, Australia), from which it was directed to a MacLab 4e computer running Chart software (version 3.5). A micromanipulator was used to set the muscle at its optimal length, and contractions were elicited with electrical stimuli. Twitch (8-V single pulse, 3-ms duration) and tetanus (8 V, 20 pulses at 90 Hz for 6 ms) responses were elicited every minute until the response fell to <50% of the maximum tetanus.

The time difference between stimulus and response and half-rise and half-decay times were also determined for the maximal twitch response.

The middle of the belly of the contralateral gastrocnemius was mounted on a stub and rapidly frozen at −40°C in methylbutane suspended in liquid nitrogen. Sections (10 μm) were cut in a cryostat at −20°C, mounted on a slide with Kaiser’s glycerol, and stained with eosin. Several sections were observed under a dissection microscope at ×33 magnification and photographed using an Olympus DP10 camera. The cross-sectional area of the largest section was calculated using Videoscan (IBM) software and used to size correct the force production data.

Muscle biochemistry. The cranic muscle was roughly sliced, immersed in liquid nitrogen, and temporarily stored at −80°C. A bead beater (BIO101 FastPrep FP120) was used to homogenize ~0.2 g of tissue in 1,000 μl of PBS in two 30-s pulses. Between pulses, the sample was kept on ice for 2 min. The resultant slurry was pipetted into a fresh Eppendorf tube and spun at 3,000 g in a tabletop microcentrifuge at 4°C for 3 min. The supernatant was divided into aliquots, stored at −80°C, and subsequently used for analysis of water-soluble proteins. The pellet was briefly washed in PBS, resuspended and incubated in a mild detergent (1% CHAPS), and respun at 3,000 g. The supernatant was divided into aliquots, stored at −80°C, and subsequently used for analysis of membrane-bound proteins.

The antioxidant potential of water-soluble and membrane-bound proteins was assessed using a commercially available colorimetric kit (Total Antioxidant Power; Oxford Biomedical Research) following the manufacturer’s instructions. The colorimetric reading was assayed on a multiplate colorimetric spectrophotometer at 490 nm. Total protein content was determined using a commercially available spectrophotometric kit (BSA protein assay; Sigma) assayed at 562 nm. The antioxidant power results are expressed per microgram of total protein.
Gene amplification, cloning, and sequencing. Genes of interest were amplified using standard PCR in up to 60-μl reactions using neat, 1:2 or 1:5 cDNA. The stringency of the reaction was adjusted for the degenerate primers by lowering the annealing temperature from 95°C to 50°C. The amplicons were identified by size after 1.8% agarose gel electrophoresis, gel extracted (Qiagen gel extraction kit), and resuspended in 30 μl of ultrapure water. The cleaned amplicons were ligated into pGEMTeasy (Promega) vectors and electroporated into 40 μl of DH5α electrocompetent Escherichia coli (200 Ω, 25 μF, 1.7 kV).

Ampicillin-resistant colonies were screened for the presence of the insert by diagnostic PCR (M13 forward and reverse primers) and plasmid DNA was isolated from positive colonies using the Big Dye 3.1 protocol (Applied Biosystems). Gel separations were performed using cycling parameters defined by the manufacturer.

Plasmid DNA was isolated from positive colonies using the Big Dye 3.1 protocol (Applied Biosystems). Gel separations were performed using cycles defined by the manufacturer. Dissociation curves. Sequence Detection Software (version 2.0, Applied Biosystems), and 600 nM forward and reverse primer. Reactions were run on a sequence detector (PRISM 7900HT, Applied Biosystems) using cycling parameters defined by the manufacturer.

Each assay (in quadruplicate) included a no-template control. All PCR efficiencies were >95%, and all the assays produced unique dissociation curves. Sequence Detection Software (version 2.0, Applied Biosystems) results were exported as tab-delimited text files and imported into Microsoft Excel for further analysis. The expression of each gene was quantified absolutely by comparison with standard curves generated from plasmid serial dilutions.

Statistical analysis. The two treatments were compared by Student’s t-test. If data failed the assumption of normality, they were compared

<table>
<thead>
<tr>
<th>Gene</th>
<th>Reference Sequence</th>
<th>Protein Sequences Used For Degenerate Primer Design</th>
<th>Forward</th>
<th>Reverse</th>
<th>Amplicon Size, bp</th>
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<tr>
<td>GPX4</td>
<td>NM_002085</td>
<td>Danio rerio (AY216591), Gallus gallus (NM_204220), Homo sapiens (NM_002085)</td>
<td>TACMGGGGTYWYGCTSTGTGAT</td>
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<tr>
<td>SOD2</td>
<td>NM_000636</td>
<td>Xenopus laevis (AY362041), Xenopus tropicalis (AAH75257), Melopsittacus undulates (AA072712)</td>
<td>CAYATHAYAYACNATHTT</td>
<td>CCARTTDAINACRTCDGADAT</td>
<td>348</td>
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<tr>
<td>CAT</td>
<td>NM_001752</td>
<td>X. laevis (AAH54964), Rana rugosa (BA83685), Mus musculus (P24270)</td>
<td>ACNGYARGATGGCCAYTGYGA</td>
<td>RTGNCCNGCDARTRTYYTCRCA</td>
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<td>PDK4</td>
<td>NM_002612</td>
<td>X. laevis (AAH59972), X. tropicalis (AAH76674), D. rerio (NP957290)</td>
<td>ATGAARCTTYATHGAYTT</td>
<td>YTGYTCRCAAGACTTYNTGCC</td>
<td>546</td>
</tr>
<tr>
<td>UCP2</td>
<td>NM_003355</td>
<td>X. laevis (AAH44682), H. sapiens (NP_073714), D. rerio (AAH75906)</td>
<td>GCCGSCCTRCAGMHCAGATG</td>
<td>GTTCTARAYCKKGTYTTYAC</td>
<td>483</td>
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<td>ATP synthase</td>
<td>N/A</td>
<td>N/A</td>
<td>TACACCAARATGARACEGGCCACCC</td>
<td>ATGTGRATRGGMCTCCACCARAC</td>
<td>460</td>
</tr>
<tr>
<td>NDI</td>
<td>NC_001807</td>
<td>X. laevis (NC_001573), Bufo woodhousii (AY220976), Ambystoma laterale (NC_006330)</td>
<td>AAATAYGCYYTAATTGGRGC</td>
<td>TATATHARTTGRTCRTADCG</td>
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GPX4, glutathione peroxidase 4; SOD2, superoxide dismutase 2; CAT, catalase; PDK4, pyruvate dehydrogenase kinase 4; UCP2, uncoupling protein 2; NDI, NADH ubiquinone oxidoreductase 1; N/A, not applicable.

if the BLAST hit score was >100. Degenerate primers were designed on the basis of conserved motifs in the corresponding protein sequences from three organisms (typically 1 or 2 amphibians and a representative from another group, e.g., a fish or a mammal; Table 1), which were SLOW aligned using EclustalW on ANGIS using the default settings. By cross-referencing to the original nucleotide sequences and only designing alternatives for the bases that varied, we were able to establish consensus sequences with low (<70) degeneracies.

<table>
<thead>
<tr>
<th>Gene</th>
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<tr>
<td>GPX4</td>
<td>AGTTCCACACTTTGGCATGCC</td>
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<td>SOD2</td>
<td>ACCAGAGCTTCTTCTGACAA</td>
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<td>GATGCCATTTGGCATGGAAA</td>
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<td>PDK4</td>
<td>AGTTTGTTTGCGGATTTCC</td>
<td>CAGCTCGGGAATACATGAG</td>
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<td>UCP2</td>
<td>AGGACCATAGGAAGCTACAA</td>
<td>GACCGCAAGGCTGAAGGAC</td>
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<td>ATP synthase</td>
<td>TGCTTCATGGGCTCGGTCGTA</td>
<td>CATTTGCTGGGCTGACCTTG</td>
</tr>
<tr>
<td>NDI</td>
<td>AGCATGCGGCTGACCTATTTCC</td>
<td>GTCACAAATGGAGCTGTTAAG</td>
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Table 2. Sequences on which degenerate primers were designed

Table 2. C. alboguttata specific primers for quantitative PCR

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<td>AACAAGCCTGGCGACAGCA</td>
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<td>GATGCCATTTGGCATGGAAA</td>
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<td>UCP2</td>
<td>AGGACCATAGGAAGCTACAA</td>
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<td>TGCTTCATGGGCTCGGTCGTA</td>
<td>CATTTGCTGGGCTGACCTTG</td>
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<tr>
<td>NDI</td>
<td>AGCATGCGGCTGACCTATTTCC</td>
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using the nonparametric Mann-Whitney U-test. The histological data were compared using analysis of covariance with animal length as the covariate. In all cases, \( P < 0.05 \) was considered significant. All statistical analysis was performed on Sigmastat software (version 2.0).

**RESULTS**

**Histology.** Six months of estivation had no significant impact on total cruralis muscle area (\( P = 0.309 \)) or pooled average fiber cross-sectional area (\( P = 0.093 \)). However, when considered in isolation, the cross-sectional area of the oxidative fibers significantly declined by 28% from 3,370 ± 800 \( \mu \text{m}^2 \) in the control frogs to 2,450 ± 200 \( \mu \text{m}^2 \) (\( P < 0.05 \)) in the estivators, indicating muscle atrophy. In contrast, the glycolytic fibers showed no significant reduction, although the 22% reduction (9,500 ± 1,700 and 7,400 ± 1,210 \( \mu \text{m}^2 \)) came very close to statistical significance (\( P = 0.055 \)). The histological data thus indicate a preferential atrophy of the oxidative fibers after estivation, but the overall gross anatomy of the muscle was relatively well preserved (Figs. 1 and 2).

**Antioxidant biochemistry.** The total antioxidant power of water-soluble and membrane-bound proteins was assessed in the gracilis muscle of control and 6-mo-estivating *C. alboguttata*. Water-soluble antioxidants in 6-mo estivators (23.8 ± 1.5 \( \mu \text{M/} \text{g} \)) matched levels in control frogs (22.3 ± 1.7 \( \mu \text{M/} \text{g}, \ P = 0.532 \)), as did membrane-bound antioxidants, which averaged 42.1 ± 7.3 and 31.5 ± 1.9 \( \mu \text{M/} \text{g} \) in estivating and control *C. alboguttata*, respectively (\( P = 0.18 \)). These data indicate maintenance of summed antioxidant potential in muscle tissue of *C. alboguttata* after 6 mo of estivation. To normalize to oxygen insult, we assumed a conservative reduction in oxygen consumption of 70%, to 30% of control values; Storey (29) reported 70–90% metabolic depression. Thus the total antioxidant power for water-soluble antioxidants was upregulated 3.5-fold: 79.3 and 22.3 \( \mu \text{M/} \text{g} \) in estivators and controls, respectively. This normalization was calculated by multiplying the estivator data by 100/30; i.e., 23.8 \times 100/30 = 79.3 \( \mu \text{M/} \text{g} \).

**Muscle mechanics.** The isometric contractile properties of the gastrocnemius muscle were determined for control and 9-mo-estivating *C. alboguttata*. The mass of the gastrocnemius (g) relative to snout-to-vent length (cm) was 0.0516 ± 0.03 and 0.0443 ± 0.09 for controls and 9-mo estivators, respectively (\( P = 0.459 \)). The absolute twitch force produced by the gastrocnemius (mN/mm\(^2\)) normalized to the snout-to-vent length (cm) was 318 ± 29 and 270 ± 51 for controls and estivators, respectively (\( P = 0.431 \); for tetanus it was 656 ± 38 and 524 ± 84, respectively (\( P = 0.186 \)).

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**Fig. 1.** Oxidative and glycolytic fibers in control (A) and 6-mo-estivating (B) *Cyclorana alboguttata* cruralis muscle. Glycolytic fibers stained light, and oxidative fibers stained dark. Cruralis muscle is a mosaic of both fiber types. Gross structure of the muscle is well preserved after 6 mo of estivation, but darker oxidative fibers are smaller. Scale bars 100 \( \mu \text{m} \).

**Fig. 2.** Cross-sectional area of oxidative and glycolytic cruralis fibers in control (A) and 6-mo-estivating (B) *C. alboguttata*. Largest fibers of both classes shrink.
Equally, when these data were reexpressed relative to gastrocnemius cross-sectional area, the isometric twitch properties (maximal force production, half-rise and half-decay times, and latency) of the 9-mo estivators immediately after release from their burrows did not differ significantly from those of the control group (see Table 4). The isometric tetanic properties of the gastrocnemius (maximal force production and tetanic-to-twitch ratio) were also maintained at control levels (see Table 4). However, the rate of fatigue of the gastrocnemius was significantly greater in the 9-mo estivators than in the controls, indicative of a loss in endurance after lengthy bouts of estivation (Fig. 3).

Expression data. No amphibian has been fully sequenced. *Xenopus tropicalis* and *Xenopus laevis*, the two best-annotated amphibian species, are members of an ancient lineage, the Archaeabatrachia. The Hyloidea, which includes Cycloraniids, are believed to have diverged from this lineage ~152,000,000 years ago (2), which seems to be close to the upper limit at which reasonable heterologous hybridization can occur (24). Consequently, degenerate primers were designed to amplify >200 bp of the *C. alboguttata* sequence (Table 1). The nucleotide sequence returned from *C. alboguttata* genes cloned in this study showed the highest similarity to amphibian and reptilian sequences and was typically 80% similar to *X. laevis* and *X. tropicalis* (Table 3).

The absolute mRNA transcript number of the seven genes was determined in control and 6-mo-estivating frogs from a fixed amount (2.5 µg) of total RNA. Estivation for 6 mo led to a reorganization of this small subset of the cruralis transcriptome (Figs. 4 and 5). Antioxidant defense gene expression was

### Table 3. Identity and functional homology of *C. alboguttata* sequence

<table>
<thead>
<tr>
<th>Gene</th>
<th>Returned nucleotide sequence</th>
<th>Highest Annotated BLAST Hit</th>
<th>Putative protein sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD2</td>
<td>SOD2, <em>X. tropicalis</em>, BC075257 (82%, 196, 2e-47)</td>
<td><em>SOD2</em>, <em>X. tropicalis</em>, AAH75257 (92%, 237, 6e-62)</td>
<td></td>
</tr>
<tr>
<td>CAT</td>
<td>CAT, wrinkled frog <em>R. rugosa</em>, AB031872 (84%, 720, 0.0)</td>
<td><em>CAT</em>, <em>R. rugosa</em>, Q9PWF7 (81%, 553, 1e-186)</td>
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<tr>
<td>PDK4</td>
<td>PDK4, African clawed toad <em>X. laevis</em>, BC041112 (81%, 274, 2e-70)</td>
<td><em>PDK4</em>, <em>X. laevis</em>, AAH76674 (95%, 899, 8e-96)</td>
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<tr>
<td>UCP2</td>
<td>UCP2, <em>X. tropicalis</em>, BC063352 (86%, 184, 1e-43)</td>
<td><em>UCP2</em>, <em>X. laevis</em>, AAH44682 (88%, 758, 1e-79)</td>
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</tr>
<tr>
<td>ATP synthase</td>
<td>ATP synthase (mitochondrial), <em>D. rerio</em>, AY393840 (89%, 507, 1e-141)</td>
<td>ATP synthase (mitochondrial), <em>Rattus norvegicus</em>, P15999 (99%, 286, 9e-77)</td>
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<tr>
<td>NDI</td>
<td>NDI, Tanganyikan wedge-snouted worm lizard <em>Geocalamus acutus</em>, AY605476 (87%, 163, 3e-37)</td>
<td>NDI, <em>Bufo woodhousii</em>, AAPH6041 (76%, 230, 8e-60)</td>
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</tbody>
</table>
modulated by 6 mo of estivation, but the effect of SOD2 differed from that of CAT and GPX4. Mitochondrial SOD2 transcription was downregulated 67% ($P < 0.05$), whereas CAT and GPX4 gene expression was maintained at close to control values ($P = 0.423$ and $P = 0.251$; Fig. 4).

The gene for substrate combustion, PDK4, was downregulated 73% after 6 mo of estivation, but this did not reach statistical significance ($P = 0.112$), probably because of the wide individual variation in the control group. The genes for the mitochondrial encoded enzymes ND1 and ATP synthase were significantly and similarly downregulated by 71% ($P < 0.05$) and 67% ($P < 0.05$), respectively. In contrast, the transcripts of the nuclear-encoded mitochondrial protein UCP2 were maintained close to control values ($P = 0.312$; Fig. 5).

**DISCUSSION**

Estivation in Cycloradinid frogs is characterized by a whole animal metabolic depression. Metabolic depression protects against muscle atrophy for at least two reasons. First, because lipid is combusted before muscle, hypometabolism functionally extends lipid stores and delays the need to combust muscle protein. Second, because a fixed proportion of oxygen respired is converted to reactive oxygen species (ROS), lowered aerobic flux will lead to absolute reductions in ROS consistent with metabolic rate (10) (with the assumption of constant uncoupling), thereby reducing atrophy associated with oxidative damage.

In the present work, we have investigated the reorganization of metabolism in estivation as indicated by a small subset of mRNA transcripts. This confirms previous work that dormancy in vertebrates is associated with a reprogrammed transcriptome (8, 27). In our experimental design, we have used three *C. alboguttata* hindlimb muscles: gracilis, cruralis, and gastrocnemius. We have elected to discuss the in vitro performance, biochemistry, and gene expression results from these muscles as if they apply interchangeably. However, we cannot completely exclude the possibility that the muscles undergo different structural and functional responses to estivation, such as those that might result from variation in fiber composition and loading history. In addition, it is conceivable, although in our opinion unlikely, that the gastrocnemius contractile data might have been significantly different had the muscles been sampled at 6 mo (consistent with the biochemistry and histology) instead of 9 mo.

**Histology and muscle performance.** In *C. alboguttata* cruralis muscle, the glycolytic fibers were relatively unaffected by 6 mo of estivation, but the oxidative fibers were significantly reduced in cross-sectional area ($P < 0.05$). Nevertheless, the reduction in glycolytic fiber area did approach significance, and if the sample size had been larger or estivation length had been increased, it might have reached significance. Fundamentally, the impact of 6 mo of estivation on *C. alboguttata* muscle appears to be more consistent with mammalian models of disuse (where oxidative fibers preferentially atrophy) than starvation (where glycolytic fibers tend to atrophy).

The present findings that the raw force data (normalized to frog length, not muscle cross-sectional area) tend to be lower after estivation (although not significantly with this sample size and estivation length) probably reflect the atrophy of individual fibers as documented by the histological data. Consequently, there may be some impact of 6 mo of estivation on whole animal locomotor performance. On the other hand, the muscle area-corrected contractile properties of the gastrocnemius are largely unchanged after 9 mo of estivation (Table 4), which is broadly in agreement with previous work on brief (3-mo) bouts of estivation in *C. alboguttata* (24) and hibernation in hamsters, *Mesocricetus auratus* (48). They indicate that, even after extended bouts of estivation, the functional capacity of the remaining actin, myosin, sarcoplasmic reticulum, and myofibrillar organelles remain largely unimpaired.

However, the significant reduction in endurance capabilities of the gastrocnemius (Fig. 3; $P < 0.05$) and the fact that the decline in tetanic force production (Table 4) approaches significance ($P = 0.087$) indicate that at 6–9 mo, contractile...
evidence for degenerative changes has accumulated in *C. alboguttata*. This evidence supports the histological data that the oxidative fibers are most affected. Nevertheless, the overall disuse response is fundamentally weaker than in clinical situations in mammals, where significant atrophy can occur in as little as 4 days of limb immobilization (25). Consequently, *C. alboguttata* represents a pertinent system to identify biochemical and gene expression correlates for decelerated muscle atrophy.

Antioxidant defense. The raw absolute “total antioxidant power” data are consistent with the activities of specific muscle antioxidants observed in the estivating spadefoot toad *Scaphiopus couchii*, in which absolute values of glutathione transferase, glutathione reductase, GPX4, and CAT were maintained during a short (2-mo) period of estivation (10). However, relative to a diminished oxygen insult, total antioxidant defense is effectively elevated threefold in estivating *C. alboguttata*.

Muscle disuse atrophy in mammals is associated with an accumulation of oxidative damage (16, 18–20) due in part to transcription of antioxidant enzymes in mammalian species in response to muscular activity. For example, unloaded mammalian muscle contains 54% less CAT (21), which is thought to contribute to ROS-induced muscle atrophy. Furthermore, experimental application of antioxidants such as vitamin E decelerates muscle atrophy in immobilized rat limbs (17). It is of interest, therefore, that, in a muscle system specifically adapted to disuse followed by rapid remobilization, relative CAT defense is endogenously upregulated. This points to CAT as an interesting candidate for the therapeutic control of muscle disuse atrophy in vertebrates.

The transcript data for control *C. alboguttata* show that different antioxidant enzymes vary widely in abundance in muscle tissue: GPX4 is abundant, mitochondrial SOD2 is rare, and CAT is intermediate.

The contrasting response within *C. alboguttata* of SOD2 vs. CAT and GPX4 transcription is of particular interest. CAT and GPX4 are representative of nuclear-encoded antioxidants, whereas SOD2 is mitochondrially encoded. A likely explanation is that as SOD2 specifically protects mitochondria from oxidative damage, it simply tracks the aerobic activity of the mitochondrion itself. Oxygen consumption is reduced ~70% in estivation, and SOD2 defense almost exactly parallels this change.

**Fuel selection.** Estivating amphibians, similar to hibernating mammals, preferentially combust lipids during dormancy (29), consistent with the sparing of lean muscle mass. In hibernating mammals, as represented by the 13-lined ground squirrels *Spermophilus tridecemlineatus*, the combustion of lipids is controlled at the biochemical level by a fivefold upregulation of PDK4 enzyme activity (5).

The present study shows a 71% downregulation in PDK4 transcripts in 6-mo estivators, although this does not reach statistical significance because of high interindividual variation. This variation may be a product of inadequate sample size (*n* = 6) or a genuine biological effect relating to 1) gender [female frogs generally tend to deposit significantly more lipid than male frogs (3)] or 2) the overall size range, which may impose allometric scaling considerations on substrate use. Nevertheless, the response of frog PDK4 to estivation contrasts markedly with that of hibernating mammals and suggests that estivators, as represented by *C. alboguttata*, use an alternative biochemical switch point.

**Energy metabolism and ROS production.** Usually, a fixed proportion (~5%) of respired oxygen is converted to ROS (1). By assessing ND1 and UCP2 transcripts, we were interested to see whether mitochondrial remodeling suggested a reduction (~5%) in the relative proportion of ROS produced. The maintenance of UCP2 transcripts at control levels suggests that the energetically costly process of uncoupling is maintained during estivation. The results for ND1, which is used as an indicator of electron transport activity and the generation of the mitochondrial proton gradient (taken together with the ATP synthase data), are consistent with a marked downregulation in electron transport activity and mitochondrial quiescence.

In addition, the similarity in the expression patterns of ND1 and ATP synthase relating to mitochondrial activity and SOD2 relating to mitochondrial antioxidant defense is noteworthy, inasmuch as they are encoded by the mitochondrial genome. The ND1, ATP synthase, and UCP2 data are consistent with the respiratory data available for overwintering hypoxic European common frogs, *Rana temporaria*. During hibernation under ice-covered ponds, *R. temporaria* exhibit no change in proton conductance but do show decreased electron transport activity (43). These results suggest common mechanisms of metabolic depression in amphibia, whether it be terrestrial estivation at 25°C or aquatic hibernation at 0°C. Given that hibernating and estivating frogs rely on finite endogenous lipid resources, reducing mitochondrial uncoupling might extend dormancy, but this is not the case.

An explanation may be that low UCP2 expression correlates with high levels of ROS production (7). The approximate maintenance of UCP2 expression may reflect a compromise to keep ROS production to a minimum, without wasting too much fuel on proton gradient dissipation. On the other hand, previous work in endotherms suggests that electron transport enzymes and UCP expression might vary between mammals and amphibia. For example, ND2, another subunit in complex I of the respiratory chain, exhibits a fourfold upregulation in the hibernating ground squirrel, *Spermophilus lateralis* (9). Similarly, in torpid hummingbirds (31) and hibernating arctic ground squirrels, *Spermophilus parryii* (4), the UCP3 muscle transcripts respond by upregulating 1.5- to 3.4- and 3-fold, respectively. UCP futile cycling generates heat, consistent with the rewarming bouts central to mammalian hibernation physiology, at cold (close to 0°C) ambient temperatures. In contrast, *C. alboguttata* is ectothermic and hypometabolic at 25°C in the laboratory. These

<table>
<thead>
<tr>
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<th>Controls</th>
<th>9-Month Estivators</th>
<th><em>P</em></th>
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<tbody>
<tr>
<td>Twitch stress, nM/mm²</td>
<td>55.9 ± 2.3</td>
<td>50.7 ± 5.1</td>
<td>NS</td>
</tr>
<tr>
<td>Tensile stress, nN/mm²</td>
<td>117.8 ± 9.2</td>
<td>98.5 ± 4.4</td>
<td>NS</td>
</tr>
<tr>
<td>Half-rise time, s</td>
<td>0.063 (0.061, 0.067)</td>
<td>0.062 (0.062, 0.064)</td>
<td>NS</td>
</tr>
<tr>
<td>Half-decay time, s</td>
<td>0.143 ± 0.006</td>
<td>0.137 ± 0.003</td>
<td>NS</td>
</tr>
<tr>
<td>Latency, s</td>
<td>0.0285 (0.0260, 0.0290)</td>
<td>0.0275 (0.0260, 0.0280)</td>
<td>NS</td>
</tr>
<tr>
<td>Twitch-to-tetanus ratio</td>
<td>0.484 ± 0.028</td>
<td>0.511 ± 0.032</td>
<td>NS</td>
</tr>
</tbody>
</table>

Normally distributed data are mean ± SE; nonnormally distributed data are expressed as medians, with 25%, 75% confidence intervals in brackets. NS, not significantly different.
contrasting thermal requirements may dominate the expression patterns of UCPs in organisms from such different taxa.

The maintenance of UCP2 compared with the depression in ND1 and ATP synthase expression could also impact the fundamental aerobic physiology of the mitochondrial respiratory chain, such as delayed muscle phosphocreatine resynthesis after exercise (22). These alterations may contribute to the present performance data showing a 32% reduction in endurance of 9-mo-estivating muscle under repeated isometric contraction (P < 0.05).

In conclusion, oxidative fibers atrophy during extended bouts of estivation in *C. alboguttata*, as assayed histologically and by in vitro contractile performance. This change is more similar to mammalian disuse than starvation is but much less pronounced. Relative to oxygen insult, antioxidant defense is bolstered, but this seems to be a reflection of the maintenance of nuclear-encoded antioxidants (CAT and GPX4), rather than bolstered, but this seems to be a reflection of the maintenance of nuclear-encoded antioxidants (CAT and GPX4), rather than mitochondrially encoded antioxidants (SOD2). Consequently, atrophy associated with oxidative damage to vulnerable, static, estivating skeletal muscle seems to be kept to a minimum by a combination of a reduction in total oxygen insult (lowering estivating skeletal muscle seems to be kept to a minimum by) a combination of a reduction in total oxygen insult (lowering absolute production of ROS after a coordinated downregulation of ND1 and ATP synthase); 2) a maintenance of UCP2 transcription (keeping the relative production of ROS to 5%), despite the associated energetic cost; and 3) a modulation of specific antioxidant enzymes. Additionally, although lipid reserves are presumably exhausted before protein is combusted in *C. alboguttata*, the biochemical switch point appears to differ from that in hibernating mammals. Although there are commonalities between estivation and hibernation at the molecular genetic, biochemical, and physiological levels, this study highlights some fundamental differences; some of these differences may be purely taxonomic in origin (PDK4), whereas others (UCP2) may reflect considerations of thermal biology.

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REFERENCES


