Myocardial performance and adaptive energy pathways in a torpid mammalian hibernator

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Heinis FI, Vermillion KL, Andrews MT, Metzger JM. Myocardial performance and adaptive energy pathways in a torpid mammalian hibernator. Am J Physiol Regul Integr Comp Physiol 309: R368 –R377, 2015. First published May 27, 2015; doi:10.1152/ajpregu.00365.2014.—The hearts of mammalian hibernators maintain contractile function in the face of severe environmental stresses during winter heterothermy. To enable survival in torpor, hibernators regulate the expression of numerous genes involved in excitation-contraction coupling, metabolism, and stress response pathways. Understanding the basis of this transition may provide new insights into treatment of human cardiac disease. Few studies have investigated hibernator heart performance during both summer active and winter torpid states, and seasonal comparisons of whole heart function are generally lacking. We investigated the force-frequency relationship and the response to ex vivo ischemia-reperfusion in intact isolated hearts from 13-lined ground squirrels (Ictidomys tridecemlineatus) in the summer (active, July) and winter (torpid, January). In standard euthemic conditions, we found that winter hearts relaxed more rapidly than summer hearts at low to moderate pacing frequencies, even though systolic function was similar in both seasons. Proteome data support the hypothesis that enhanced Ca2+ handling in winter torpid hearts underlies the increased relaxation rate. Additionally, winter hearts developed significantly less rigor contracture during ischemia than summer hearts, while recovery during reperfusion was similar in hearts between seasons. Winter torpid hearts have an increased glycogen content, which likely reduces development of rigor contracture during the ischemic event due to anaerobic ATP production. These cardioprotective mechanisms are important for the hibernation phenotype and highlight the resistance to hypoxic stress in the hibernator.

hibernation; isolated heart; force-frequency relationship; sarcoplasmic reticulum; Ca2++; ischemia-reperfusion injury; Langendorff; season; torpor; glycolysis; glycolgenolysis

MAMMALIAN HIBERNATORS survive tremendous environmental challenges during winter by entering a transient heterothermic state, torpor (TOR). During TOR, body temperature drops to near-ambient level, yet cardiac performance is sufficiently preserved to enable life-sustaining circulation (reviewed in Ref. 2). To support this winter torpid state, hibernators orchestrate seasonal changes in the expression of genes involved in cardiac muscle metabolism, excitation-contraction coupling, stress responses, and other pathways that are thought to collectively confer resistance to cold temperature-mediated heart pump dysfunction (8, 28). These seasonal transitions in gene expression are of potential medical interest, as mechanisms underlying the remarkable stress resistance of hibernators may lead to new approaches toward remediation of heart disease in nonhibernators, including humans. Despite this potential value, relatively few studies have been conducted to elucidate hibernator cardiac function in both the summer and winter seasons.

During TOR, hibernator hearts demonstrate a physiological robust contraction-relaxation cycle, albeit at drastically fewer beats per minute under severe hypothermic conditions, that depresses contractile function and renders nonhibernator hearts highly arrhythmic (11, 23). Hibernators are also known to be highly resistant to hypoxic stress (reviewed in Ref. 20). Sarcoplasmic reticulum (SR) Ca2+ uptake capability is greater in cardiac muscle from hibernators than nonhibernators, and cardiac muscle from hibernators is capable of maintaining low diastolic Ca2+ levels at temperatures well below those tolerated by nonhibernator myocytes (35). In particular, hibernator myocardium from the winter torpid state has increased gene expression of the SR Ca2+-ATPase (SERCA2), as well as decreased expression of the SERCA2 inhibitor phospholamban (PLN) (8, 28, 53), relative to hibernator hearts during the summer months. This seasonal increase in the SERCA-to-PLN ratio is likely highly important for enabling contractility during TOR. Because SR Ca2+ uptake is impaired in human heart failure (27, 29, 47), the ability of hibernators to seasonally regulate SR function is of significant interest. Therefore, a comparison of hibernator cardiac function in the summer active and winter torpid states is likely to identify the functional significance of altered gene expression. Studies of isolated papillary muscle strips and cardiac myocytes from hibernator hearts have shown greater reliance on SR Ca2+ handling for contractility by myocardium from winter torpid than summer active ground squirrels (31, 32, 53). Seasonal changes in cardiac Ca2+ handling, however, have not been evaluated in the physiologically relevant context of the intact heart.

We investigated whether whole heart contractile performance is influenced by season in hibernators. Owing to the increased SR function in winter torpid hibernators, we hypothesized that systolic and diastolic performance would be enhanced in the hearts of winter torpid ground squirrels compared with summer active animals at 37°C. We isolated whole hearts from ground squirrels in summer (July) and winter (January) and determined the force-frequency relationship (FFR) by Langendorff-mode perfusion. By subjecting each heart to progressively increased stimulation frequencies, we stressed the heart’s ability to sequester Ca2+ at each beat, revealing SR contributions to contractile function. Each heart was then subjected to ischemia-reperfusion injury to determine whether seasonal changes in Ca2+ handling or metabolism conferred additional resistance to ischemic damage in torpid hearts. To identify mechanisms underlying differences in function be-
between summer active and winter torpid ground squirrel hearts, we compared protein expression profiles between torpid and active hibernator hearts. Although it is well established that hibernators are highly resistant to hypoxic stress, most such comparisons have been made between hibernator and nonhibernator hearts, rather than between hibernator hearts during summer and winter. Thus an additional rationale for this study was to test whether hypoxia resistance is a general property of hibernators or whether it is also regulated during TOR.

**METHODS**

**Animal care and husbandry.** Animal husbandry and experiments were approved by the Institutional Animal Care and Use Committee of the University of Minnesota (protocols 0805A34502 and 1103A97712). Thirteen-lined ground squirrels (*Ictidomys tridecemlineatus*) of both sexes were trapped from the wild in the spring, dewormed, and housed individually at the University of Minnesota-Duluth. During summer months, squirrels were housed at 22°C with a 12:12-h light-dark cycle, with water and standard rodent chow available ad libitum. For induction of TOR, squirrels were transferred to a dark cold-room at 4°C and food was removed. Induction and maintenance of TOR were monitored by the sawdust method. Summer active ground squirrels were studied in July, and all winter torpid ground squirrels were studied in January. All ground squirrels studied in January were taken from the torpid state [4.1 ± 1.1 days since last interbout arousal (IBA)].

**Langendorff heart preparation.** Squirrels were anesthetized with isoflurane gas (5% induction, 2% maintenance, balance pure O2). Anesthetic depth was monitored by toe pinch. Heparin (700 IU ip) was administered after anesthetic induction but prior to surgery. Upon loss of reflex, the chest was entered and the heart was surgically removed to a dish of ice-cold Krebs-Henseleit buffer (KHB; in mmol/l: 118 NaCl, 4.7 KCl, 1.2 NaH2PO4, 1.2 MgSO4, 2.5 CaCl2, 0.5 NaEDTA, 15 glucose, 25 NaHCO3, 0.5 Na pyruvate). Lungs and pericardial fat were trimmed away to reveal the aortic arch. Before cannulation, each heart was briefly moved to a nearby scale and pericardial fat were trimmed away to reveal the aortic arch. Before cannulation, each heart was evaluated in two phases (Fig. 1A). Phase 1 consisted of 5 min of equilibration at a pacing frequency of 6 Hz. After equilibration, the pacing frequency was decreased from 6 Hz to 3 Hz in 1- and 0.5-Hz increments (6, 5, 4.5, 4, 3.5, and 3 Hz). After 3 Hz, hearts were returned to 6 Hz; then the pacing frequency was increased to 10 Hz in 1-Hz increments. At each new pacing frequency, the heart was allowed sufficient time to achieve stable performance, between 30 and 90 s, before the next frequency step. After performance at 10 Hz stabilized, the pacing frequency was returned to 6 Hz. At <4 Hz, it became difficult to reliably pace hearts at the defined frequency, so 4 Hz was the lowest pacing frequency analyzed in this study.

Hearts were initially perfused with KHB containing 0.5 mM pyruvate to facilitate collection of pacing data. At the end of the pacing challenge phase, hearts were perfused with KHB lacking pyruvate and reequilibrated at 6 Hz for 10 min. KHB lacking pyruvate was necessary for phase 2, ischemia-reperfusion, to avoid the ischemia-protective effects of pyruvate (18, 30). After reequilibration in KHB lacking pyruvate, the pacing electrode was inactivated and perfusate flow was stopped for 25 min to cause global myocardial ischemia. After ischemia, hearts were reperfused for 60 min. At 8 min after reperfusion, the pacing electrode was reactivated at 6 Hz and remained active until the end of the experiment.

Upon completion of ex vivo perfusion, each heart was removed from the Langendorff apparatus. A portion of the LV free wall was excised, frozen in liquid nitrogen, and stored at −80°C until use. Tissue samples were pulverized with a liquid nitrogen-cooled mortar and pestle, resuspended in RIPA buffer [50 mmol/l Tris, 150 mmol/l NaCl, 1 mmol/l EDTA, and 0.5% (wt/vol) SDS] containing protease inhibitors (aprotinin, leupeptin, and pepstatin, each at 0.001 mg/ml, and 1 mmol/l phenylmethylsulfonyl fluoride), sonicated briefly, and sedimented at 15,000 g for 2 min. The protein concentration of the cannulated heart was perfused in the Langendorff mode (Radnoti) in a two-phase protocol (see below) at a constant pressure of 75 mmHg. The KHB perfusate was bubbled with 5% CO2-95% O2, and temperature was maintained at 37°C by water-jacketed tubing for all experiments. Both atria were removed, and left ventricular (LV) pressure was measured by a pressure transducer connected to a water-filled balloon catheter inserted into the LV. The pacing frequency was artificially controlled by an electrode placed at the root of the right atrium.

**Pacing challenge and ischemia-reperfusion.** While perfused on the Langendorff apparatus, each heart was evaluated in two phases (Fig. 1A). Phase 1 consisted of 5 min of equilibration at a pacing frequency of 6 Hz. After equilibration, the pacing frequency was decreased from 6 Hz to 3 Hz in 1- and 0.5-Hz increments (6, 5, 4.5, 4, 3.5, and 3 Hz). After 3 Hz, hearts were returned to 6 Hz; then the pacing frequency was increased to 10 Hz in 1-Hz increments. At each new pacing frequency, the heart was allowed sufficient time to achieve stable performance, between 30 and 90 s, before the next frequency step. After performance at 10 Hz stabilized, the pacing frequency was returned to 6 Hz. At <4 Hz, it became difficult to reliably pace hearts at the defined frequency, so 4 Hz was the lowest pacing frequency analyzed in this study.

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**Fig. 1.** Protocol schematic and individual pressure traces at 37°C. A: schematic of Langendorff-isolated heart protocol. B: representative left ventricular (LV) pressure traces from summer active and winter torpid ground squirrel hearts at 4, 6, and 10 Hz. Summer and winter hearts developed similar systolic LV pressures, and relaxation was faster at low frequency in winter hearts.

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**Table:**

A

<table>
<thead>
<tr>
<th>Equilibrate</th>
<th>Pace Hearts</th>
<th>Re-Equilibrate</th>
<th>Ischemia Unpaced</th>
<th>Reperfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 Hz</td>
<td>4-10 Hz/step</td>
<td>6 Hz</td>
<td>25 Minutes</td>
<td>60 Minutes</td>
</tr>
<tr>
<td>5 Minutes</td>
<td>&lt;1 minute/step</td>
<td>10 Minutes</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**B**

![Representative LV Pressure Traces](image-url)

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**Details:**

- **A:** Table layout.
- **B:** Graph showing representative pressure traces.
- **Figure 1:** Protocol schematic and pressure traces.
heart homogenate supernatant was determined by the bicinchoninic acid method (Thermo Scientific).

Animal preparation. For proteome analysis, animals were housed as described above. Animals in TOR were collected after ≥3 days in a TOR bout and showed no visible signs of arousal. At the time the animals were euthanized, rectal temperatures were taken to verify torpid state (6–8°C body temperature). For the active time point, animals were euthanized at each collection point. All animals were fully anesthetized with isoflurane and then euthanized by decapitation. The pericardium was removed from around the heart, and the heart was removed from the animal and halved sagittally to include atrium and ventricle. Heart dissection was performed on ice, and dissected heart pieces were rinsed with PBS and rapidly flash-frozen in liquid nitrogen. The time from decapitation to sample freezing was <10 min. Tissue was stored at −80°C.

Protein extraction, proteolytic digestion, and labeling with isobaric tags for relative and absolute quantitation. One-half of the heart from each animal was used for protein sample preparation. Frozen tissue was ground to a fine powder on liquid nitrogen using a mortar and pestle. Tissue powder was brought up in iTRAQ lysis buffer [7 M urea, 2 M thiourea, 0.5 M triethylammonium bicarbonate (pH 8.5), 20% acetonitrile, 4 mM tris(2-carboxyethyl)phosphine, and Roche PhosSTOP]. Probe sonication was used to lyse samples (30% amplitude, 7-s pulse). Samples were then put into PCR tubes and run in the Barocycler to efficiently isolate membrane proteins and hydrophobic proteins. Samples were transferred to a new tube, methyl methanethiosulfonate was added to a final concentration of 8 mM, and the samples were incubated at room temperature for 15 min. Bradford assay was used to determine protein concentration. To examine seasonal expression changes independent of sex effects, for each time point, three samples consisting of pooled tissue from one male and one female animal were analyzed; this limitation decreased sample size in this study. Fifty micrograms of protein from the male and 50 μg of protein from the female were combined and digested in trypsin (1:35 trypsin-total protein) overnight at 37°C. After trypsin digestion, samples were cleaned with a 4-ml Extract-Clean C18 solid-phase extraction cartridge (Grace-Davidson, Deerfield, IL). Samples were vacuum-centrifuged to dryness and resuspended in dissolution buffer (0.5 M triethylammonium bicarbonate, pH 8.5) to a final concentration of 2 μg/μl. Three micrograms of each sample were loaded onto the linear trap quadrupole for a quality control run. Total ion chromatograms were produced to identify blood contamination and determine quality of each sample. For each iTRAQ 8-plex, 40 μg of each sample were labeled with iTRAQ reagent according to the manufacturer’s protocol (Ab Sciex, Foster City, CA). Protein lysates were iTRAQ-labeled, with one label corresponding to each time point. The lysates from two time points were split and labeled with two separate

Table 1. Animal characteristics

<table>
<thead>
<tr>
<th></th>
<th>Summer (n = 10)</th>
<th>Winter (n = 9)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body wt, g</td>
<td>211.54 ± 6.98</td>
<td>156.44 ± 13.58</td>
<td>0.0017</td>
</tr>
<tr>
<td>Heart wt, mg</td>
<td>896 ± 33.93</td>
<td>932 ± 31.13</td>
<td>0.4514</td>
</tr>
<tr>
<td>Tibia length, mm</td>
<td>34.30 ± 0.21</td>
<td>33.89 ± 0.42</td>
<td>0.3833</td>
</tr>
<tr>
<td>Heart wt/body wt, g/mg</td>
<td>4.30 ± 0.27</td>
<td>5.96 ± 0.36</td>
<td>0.0017</td>
</tr>
<tr>
<td>Heart wt/tibia length, mg/mm</td>
<td>26.17 ± 1.11</td>
<td>27.46 ± 1</td>
<td>0.4089</td>
</tr>
<tr>
<td>Body temperature, °C</td>
<td>6.96 ± 0.28</td>
<td>6.98 ± 0.20</td>
<td>0.3317</td>
</tr>
</tbody>
</table>

Values are means ± SE. Ground squirrels studied in summer (July) were significantly heavier than those studied in winter (January). Heart weights and tibia lengths were not significantly different, but this was due to differences in body weight, as heart weight-to-tibia length ratios were not significantly different between groups. P values were determined by unpaired 2-tailed t-test.
iTRAQ labels for technical replicates. After the samples were labeled, they were multiplexed together and vacuum-dried. The multiplexed samples were cleaned with a 4-ml Extract-Clean C18 solid-phase extraction cartridge, and the eluate was dried in vacuo.

Peptide liquid chromatography fractionation and mass spectrometry. The iTRAQ-labeled samples were resuspended in buffer A [20 mM ammonium formate (pH 10) in 98:2 water-acetonitrile] and fractionated offline by high-pH C18 reverse-phase chromatography (52). A HPLC (Prominence, Shimadzu, Columbia, MD) with a C18 XBridge column (150 mm × 2.1 mm internal diameter, 5-μm particle size; Waters, Milford, MA) was used. Buffer B contained 20 mM ammonium formate (pH 10) in 10:90 water-acetonitrile. The flow rate was 200 μl/min, with a gradient from 2 to 35% buffer B over 60 min, followed by 35–60% over 5 min. Fractions were collected every 2

Fig. 4. Expression of Ca^{2+}-handling proteins in torpor (TOR) relative to the summer active time point in August (AUG). Protein expression of ATP2A2 and SLCA8A1 significantly increased, while protein expression of ryanodine receptor type 2 (RYR2) and phospholamban (PLN) did not significantly decrease, in TOR. Protein expression of ATP2A2 and SLCA8A1 in TOR (n = 3) was significantly different from that in AUG (n = 3) as determined by Student’s t-test: *P < 0.05.

min, and UV absorbances were monitored at 215 and 280 nm. Peptide-containing fractions were divided into two equal numbered groups, “early” and “late.” The first early fraction was concatenated with the first late fraction, and so on. Concatenated samples were dried in vacuo and resuspended in load solvent (98:2:0.01 water-acetonitrile-formic acid), and 1- to 1.5-μg aliquots were run on a Velos Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA) as described previously (36), with the exception that the high-energy collisional dissociation activation energy was 20 ms.

Glycogen content assay. For the glycogen assay, animals were housed as described above. Animals in TOR were collected after ≈3 days in a TOR bout and showed no visible signs of arousal. At the time of euthanization, rectal temperatures were taken to verify torpid state (6–8°C body temperature). Animals collected for the IBA collection point aroused naturally, were awake and active, and showed coordinated body movement. Rectal temperatures recorded at euthanization showed an active body temperature between 35 and 37°C. TOR and IBA animals were collected in January and February, when TOR bouts are the longest. Animals were collected for the summer active time point in August (AUG); they were awake and active and showed an active body temperature of 35–37°C. Animals were fully anesthetized with isoflurane and then euthanized by decapitation. The pericardium was removed from around the heart, and the heart was removed from the animal and halved sagitally to include atrium and ventricle. Heart dissection was performed on ice, and dissected heart pieces were rapidly flash-frozen in liquid nitrogen. The time from decapitation to sample freezing was <10 min. Tissue was stored at −80°C. Ten micrograms of tissue from each sample were ground to powder in liquid nitrogen and homogenized in 100 μl of water on ice. Homogenates were boiled for 5 min to inactivate enzymes and stored at −20°C. Glycogen assay was performed according to the manufacturer’s protocol, with 12.5 μl of water added to each well, and included two replicates and a glucose background control (catalog no. MAK016, Sigma Aldrich, St. Louis, MO).

Data collection and statistics. Langendorff data were collected using LabChart 6 (ADInstruments) and analyzed using Prism 5 (GraphPad). Statistical significance was evaluated by two-way analysis of variance with Bonferroni’s post test or two-tailed t-test as

Fig. 3. Winter hearts exhibit decreased times to peak contraction, times to 50% relaxation, and exponential time constant of pressure decay (τ). A: at low to moderate stimulation frequencies, times between 50% and peak contraction were lower in winter (n = 10) than summer (n = 9) hearts. B: at low to moderate simulation frequencies, times to 50% relaxation were lower in winter hearts. C: τ was lower in winter hearts. *P < 0.05; **P < 0.01; ***P < 0.001 (by Bonferroni’s post test).
appropriate. $P < 0.05$ was considered significant. The time constant of pressure decay ($\tau$) was obtained using LabChart 7 software (ADInstruments) and the Weiss method $[P(t) = Ae^{-t/\tau} + B]$, with a sampling range that ended at end-diastolic pressure for the beat. For determination of rigor, the initiation point of rigor was manually determined using the time at which LV pressure rose above 8 mmHg. This rule was applied prior to analysis as an estimate of the time each heart’s LV pressure rose above preload levels.

For proteome analysis, Microsoft RAW files were imported into GalaxyP and searched against a customized ground squirrel database generated from the National Center for Biotechnology Information 13-lined ground squirrel genome merged with our RNA.
sequencing-derived protein sequences and the contaminants database. Files were processed into peak lists using ProteinPilot software (AB Sciex, Framingham, MA) for protein and quantification analysis. All peptides were identified with ≥95% confidence and <1% global false discovery rate between the three runs. Relative quantification was determined by ProteinPilot in a normalized log10-based relative iTRAQ ratio format, with the iTRAQ label corresponding to AUG as the reference denominator. Protein expression ratios were calculated in the Protein Alignment Template (AB Sciex).

RESULTS

Summer and winter ground squirrel characteristics. Body and heart weight characteristics of the summer and winter ground squirrels used for this study are summarized in Table 1. Body weight was significantly greater in summer than winter squirrels. Heart weight and body size (tibia length) were not significantly different between seasons. Although the heart weight-to-body weight ratio was higher in winter than summer, this was due to greater body weight in summer animals, rather than any difference in heart weight. Heart weight-to-tibia length ratios were not different between summer and winter. Absolute heart weights were not significantly different between groups.

LV function. Representative individual traces of LV pressure at 37°C are shown in Fig. 1. The magnitude of contraction (LV developed pressure) was similar at all stimulation frequencies. Pressure decay was more rapid in hearts from winter ground squirrels at low (4 Hz) and moderate (6 Hz) pacing frequencies. There was no difference in relaxation at high (10 Hz) pacing frequency (Fig. 1 and Fig. 2, B and D).

The systolic performance of perfused hearts isolated from summer and winter ground squirrels was very similar across a wide range of pacing frequencies (Fig. 2). Hearts from summer and winter squirrels underwent a negative-staircase FFR (Fig. 2A). LV developed pressures were not significantly different in hearts from winter squirrels (P = 0.0606 for season effect, by two-way ANOVA). Similarly, LV end-diastolic pressures were not significantly different between hearts from summer and winter animals from 4- to 10-Hz pacing frequency and in both groups steadily increased with each frequency step above 6 Hz (Fig. 2B). Maximal first derivatives of pressure (dP/dt max) (Fig. 2C) were not significantly different between seasons, but minimal dP/dt (dP/dt min) was significantly greater in winter hearts (P < 0.0001 for season main effect, by two-way ANOVA), indicating significantly faster relaxation on a beat-to-beat basis across all pacing frequencies. Winter hearts had significantly shorter times of pressure rise (Fig. 3A) and relaxation (Fig. 3B). τ was slightly, but significantly, lower in winter hearts at most pacing frequencies (Fig. 3C).

Proteomic analysis. To pursue the mechanism by which winter torpid hearts are able to significantly reduce times of pressure rise and relaxation, we examined cardiac proteomic data collected from ground squirrel hearts throughout the circannual cycle. Notable results from this analysis are described in Fig. 4. We found that protein expression of several key enzymes involved in Ca2+ handling was altered in torpid hearts relative to summer active hearts. In particular, the expression of SERCA2 (or ATP2A2) and NCX1 (or SLC8A1) is significantly increased in TOR relative to AUG while expression of ryanodine receptor type 2 (RYR2) and PLN was not significantly decreased in TOR relative to AUG.

Ischemia-reperfusion. After reequilibration for 10 min with KHB lacking pyruvate, each heart was subjected to 25 min of no-flow ischemia followed by 60 min of reperfusion. Traces of LV pressure during the final 20 min of ischemia from each summer and winter heart are overlaid in Fig. 5. From these traces, it is evident that summer and winter hearts exhibit markedly different behavior during ischemia: summer hearts enter rigor more rapidly and reach higher pressures than winter hearts. Typical behavior of an isolated (summer) heart during ischemia-reperfusion is shown in the inset: upon onset of ischemia, contractile force declines rapidly, and hearts enter a relaxed phase. As ATP is gradually depleted from the myocardium, an ischemic isolated heart enters a progressively contracted rigor state (38). Upon reperfusion, hearts are arrhythmic and do not recover normal rhythm for several minutes. Winter hearts either entered a slight contracture phase before reperfusion or did not enter contracture at all. This behavior is quantified in Fig. 5, B–D: both summer and winter hearts reached similarly low pressures prior to the onset of rigor (Fig. 5B). Summer hearts attained significantly higher maximal pressures than winter hearts during ischemia (Figs. 5C and 6B) and entered contracture sooner than winter hearts (Fig. 5D). Despite these differences in heart function during ischemia, both groups recovered most of their contractile function soon after perfusion was resumed (Fig. 6A), and end-diastolic pressures were not different during reperfusion (Fig. 6B). There was no significant correlation between the maximal pressure achieved during ischemic contracture by a winter heart and the time since each winter animal’s most recent IBA (Fig. 6C).

To determine if winter torpid hearts were able to resist ischemic contracture due to increased glycogen stores in the heart, we performed a glycogen assay on cardiac tissue from several time points, including AUG, OCT, TOR, and IBA. We found that cardiac glycogen content was lowest in the summer
active period and rose through the fall to TOR and IBA, showing peak content in torpid animals (Fig. 7).

**DISCUSSION**

Mammalian hibernators undergo remarkable adaptations in their physiology during the transition from high activity in summer to TOR in winter (2). As hibernators are highly resistant to environmental stress, the changes in gene expression that underlie this seasonal transition are of great potential medical interest (12). To understand the physiological role served by seasonal alterations in myocardial Ca²⁺ handling and metabolism, we evaluated the FFR and ischemia-reperfusion performance in the intact isolated hearts of active and torpid ground squirrels. Prior efforts to evaluate season-dependent function in hibernator cardiac tissue utilized isolated cardiac myocytes or papillary muscle strips (44). Although these studies have been highly informative into cellular mechanisms underlying contractility during TOR, it remained unclear whether whole-organ performance would reflect those findings. Our main new finding that ischemia-induced myocardial contracture varied significantly by season may have important implications for the treatment of ischemic injury in nonhibernators.

Whole heart relaxation performance in winter torpid hearts at 37°C is superior to that in summer active hearts, consistent with rapid diastolic Ca²⁺ sequestration and improved SR Ca²⁺-handling mechanisms in torpid hibernator myocytes (Fig. 4) (53). For relaxation of cardiac muscle to occur, intracellular Ca²⁺ must decrease to allow for Ca²⁺ release from the myofilaments. Ca²⁺ transport out of the cytosol occurs by four pathways involving SR Ca²⁺-ATPase (ATP2A2), sarcolemmal Na⁺/Ca²⁺ exchange (SLC8A1), sarcolemmal Ca²⁺-ATPase (ATP2B), or mitochondrial Ca²⁺-unimport (6). Increased protein expression of ATP2A2 and SLC8A1 in TOR relative to AUG correlates with our previous transcriptomic analysis (8). These two mechanisms account for removal of 98% of intracellular Ca²⁺ in rabbit ventricular myocytes (5). We found no significant differences in RYR2 expression between the active and torpid states. All these factors contribute to the increased removal of Ca²⁺ from the cytoplasm and would result in faster relaxation. These findings are similar to previous findings, including those reported by Yatani et al. (53), except Yatani et al. found significantly decreased protein expression of PLN. Both our study and

![Graph](https://example.com/graph1.png)

Fig. 6. Recovery from ischemia-reperfusion injury was similar in summer and winter hearts. **A**: LVDPs were similar in summer (n = 8) and winter (n = 9) hearts throughout ischemia-reperfusion injury. No significant differences were found. **B**: LVEDPs were similar during reperfusion, suggesting no differences in severity of ischemia-reperfusion between groups. LVEDPs were higher in summer than winter hearts at 20 and 25 min of ischemia. ***P < 0.001 vs. winter torpid (by 2-way ANOVA with Bonferroni’s post test). **C**: maximal ischemia pressures of winter hearts were compared with each heart’s time since the most recent interbout arousal (IBA). No correlation between severity of ischemic contracture and IBAs was found (R² = 0.1066, P = 0.3911).
previous work generally support increased removal of cyto-
plasmic Ca$^{2+}$, leading to improved relaxation in hibernator
hearts during the winter season.

Our finding that the FFR is negative in both summer and
winter intact hearts is in apparent conflict with prior findings in
papillary muscle strips, which showed divergent FFR behavior
between seasons (31, 44). This difference may be due to the
pacing frequency range used in the earlier studies, 0.1–2 Hz,
which, although relevant to the torpid state, is well below the
normal heart rate of an active ground squirrel (5–7 Hz). The
present study of whole isolated hearts over a more exten-
sive and physiologically relevant frequency range shows that
the FFR of the ground squirrel, independent of season, is more
similar to the FFR of the rat than of larger mammals (40, 43).
The similarity in FFR between active and torpid ground squir-
rel hearts is intriguing, given that contractile function of the
torpid heart is more dependent on SR Ca$^{2+}$ flux than summer
active cardiac tissue (31, 32) and that active summer hibernator
heart has a far greater SR Ca$^{2+}$ uptake capability than the
nonhibernator heart (35, 37). Given that systolic and diastolic
function were highly similar between active and torpid isolated
hearts, we hypothesize that enhanced SR Ca$^{2+}$ reuptake in the
torpid heart is required for hypothermia tolerance and mainte-
nance of low diastolic Ca$^{2+}$, rather than alteration of systolic
contractile function. The finding that intrinsic organ-level sys-
tolic function is similar between active and torpid myocardium
suggests that decreased energy expenditures during TOR are a
product of a much reduced heart rate, rather than intrinsic
differences in contractile force.

Hypoxia resistance in hibernators has long been of interest,
and numerous studies have compared hypoxia responses be-
tween hibernators and nonhibernators (9, 10, 22, 39, 41),
evaluated surrogate measures of cardiac function during hyp-
oxia in summer and winter (10, 39, 42), or compared hypoxia
responses in other organs (15, 24, 33). To determine the
functional importance of seasonal changes in gene and protein
expression, however, a direct functional comparison of one
hibernator species between seasons is necessary. Our findings
show that summer active and torpid hearts diverge significantly
in the development of contracture during ischemia. Hearts
isolated from active ground squirrels rapidly enter contracture, and torpid hearts dramatically resist this effect.
This is direct organ-level functional evidence in keeping with
the known marked seasonal variations in metabolic function
that underlie the ability of torpid hearts to prevent or limit
ATP depletion that leads to contracture during bouts of ischemia (38). Our results are of particular interest, given
recent evidence demonstrating that woodchucks similarly
exhibit seasonal resistance to ischemic injury without pre-
conditioning (51).

Myocardial ischemia, and accompanying loss of O$_2$, makes
the heart dependent on anaerobic energy production to supply
energy for metabolic processes. Anaerobic energy production
largely depends on the availability of adequate glycolytic
substrates, with the ischemic heart greatly accelerating glyco-
genolysis at the onset of ischemia (4). In our comparison of
the hibernator heart with the summer active heart, we found a
differential response to no-flow ischemia. Summer hearts at-
tained significantly higher maximal pressures than winter
hearts during ischemia and entered contracture sooner than
winter hearts. Diminished ischemic contracture in torpid hearts
supports prior results that indicate that torpid hibernator hearts
are primed to utilize glucose (10, 14). Additionally, we report
that the glycogen content of torpid hearts is significantly
increased relative to summer hearts.

This increase in cardiac glycogen content is similar to that
observed during fasting in nonhibernators (3). These studies
also showed that increased glycogen content results in less
injury and improves recovery following no-flow ischemia in
isolated hearts from nonhibernators (3, 19, 26, 48). In further
comparisons of hearts from fasted and fed rats under no-flow
ischemia, it was found that fasted hearts were better protected
from ischemic injury and had a lower lactate-to-pyruvate ratio
and increased glycogen utilization (46). These and other stud-
ies also found that ischemic contracture begins when glycogen
breakdown stops and the rate of glucose uptake decreases.
These data indicate that the ischemic heart is better preserved
as long as glycogen is present and available for energy produc-
tion (13). Our observation of higher glycogen stores in
torpid hearts suggests that hibernators are able to replenish
glucose and glycogen stores from glycerol liberated from fatty
acids during the arousal periods. Galster and Morrison (25)
found that glucose-equivalent amounts of glycerol are mobi-
lized during the periodic arousals to restore glucose reserves
and that the majority of these substrates came from fatty acids,
while the rest comes from proteins from urea and ammonia in
urine. They also found that glucose utilization is restricted
during arousals, but not during hibernation. This is further
supported by our previous findings that glucose that enters the
heart during arousal remains largely intact, as evidenced by a
lack of labeled metabolites derived from the tricarboxylic acid
cycle (1). Although we observed a decreased glycogen content
in the heart during the IBA, this could be due to the timing of
collection of the IBA animals. Torpid animals are collected 3
days into a TOR bout, when replenished glucose and glycogen
levels remain high, and the IBA animals are collected upon full
arousal. This likely does not allow time for glycogen stores to
be replenished. It would be interesting in future work to assay
glycogen content over the time course of the TOR-arousal
cycle to more clearly look at changing glycogen levels.

Another mechanism proposed for the differential response to
ischemic events in the hearts of fasted vs. fed animals is a
lower cytosolic redox state in fasted animals. The redox state
can profoundly affect the rate of glycolysis by inhibiting key
regulatory enzymes such as GAPDH (34, 45). A lower redox
state in hearts from fasted animals would result in less inhibi-
tion of GAPDH, increased glycolysis (but not necessarily
glucose oxidation), increased glycogen utilization, less accum-
ulation of glycolytic intermediates, and greater anaerobic
ATP production. An accumulation of glycogen is also ob-
served in the ischemic heart disease hibernating myocardium,
where a deregulation of glycogen metabolism, from repeated
short-term ischemic events, is thought to be related to stimu-
lation of glucose uptake for glycogen synthesis (7, 17, 21, 50).
The accumulation of glycogen is also found in unloaded
myocardium and in the fetal heart, suggesting that all these
conditions, including hibernation, induce a reliance on glucose
for energy provision (16, 49). Increased glycogen stores may
provide the additional glycolytic substrates during ischemia
and reperfusion. This would allow for increased ATP produc-
tion and likely result in maintaining Ca$^{2+}$ homeostasis, result-
ing in less injury and delayed entry into contracture. Resistance

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to ischemia and hypothermia during TOR is therefore likely
due to the combined effects of metabolic and Ca\textsuperscript{2+}
transport remodeling.

**Perspectives and significance.** Mammalian hibernators have
the unique capability to resist physiological stresses, such as
hypothermia, arrhythmia, and ischemic injury, that would se-
verely injure nonhibernators. Given the impact of these stresses
on human morbidity and mortality, it is of great clinical interest
to understand the mechanisms by which hibernators can resist
injury and support cardiac function under adverse conditions.
With this work we demonstrate that resistance to injury is
seasonally regulated in hibernators, corresponding with sea-
sonal changes in Ca\textsuperscript{2+}-handling gene expression and carbohy-
drate storage. The regulated proteomic and metabolic changes
we observed are therefore useful for informing therapeutic
development and improving human clinical outcomes.

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No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**

F.I.H., K.L.V., M.T.A., and J.M.M. developed the concept and designed the
research; F.I.H., K.L.V., M.T.A., and J.M.M. analyzed the data; F.I.H., K.L.V., M.T.A.,
and J.M.M. interpreted the results of the experiments; F.I.H. and K.L.V. prepared the
figures; F.I.H. and K.L.V. drafted the manuscript; F.I.H., K.L.V., M.T.A.,
and J.M.M. edited and revised the manuscript; F.I.H., K.L.V., M.T.A., and J.M.M.
approved the final version of the manuscript.

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