

1 **Regulation of succinate-fueled mitochondrial respiration in liver and skeletal muscle of**
2 **hibernating thirteen-lined ground squirrels.**

3

4 Short title: Mitochondrial regulation in hibernation.

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1 **Summary**

2 Hibernating ground squirrels (*Ictidomys tridecemlineatus*) alternate between two distinct
3 metabolic states throughout winter: torpor, during which metabolic rate (MR) and body
4 temperature (T_b) are considerably suppressed, and interbout euthermia (IBE), during which MR
5 and T_b briefly return to euthermic levels. Previous studies showed suppression of succinate-
6 fueled respiration during torpor in liver and skeletal muscle mitochondria; however, these studies
7 used only a single, saturating succinate concentration. Therefore, they could not address whether
8 mitochondrial metabolic suppression occurs under physiological substrate concentrations or
9 whether differences in the kinetics of mitochondrial responses to changing substrate
10 concentration might also contribute to mitochondrial metabolic regulation during torpor. The
11 present study confirmed that succinate oxidation is reduced during torpor in liver and skeletal
12 muscle at 37°C and 10°C over a 100-fold range of succinate concentrations. At 37°C, this
13 suppression resulted from inhibition of succinate dehydrogenase (SDH), which had a greater
14 affinity for oxaloacetate (an SDH inhibitor) during torpor. At 10°C, SDH was not inhibited,
15 suggesting that SDH inhibition initiates but does not maintain mitochondrial suppression during
16 torpor. Moreover, in both liver and skeletal muscle, mitochondria from torpid animals
17 maintained relatively higher respiration rates at low succinate concentrations, which reduces the
18 extent of energy savings that can be achieved during torpor but may also maintain mitochondrial
19 oxidative capacity above some lower critical threshold, thereby preventing cellular and/or
20 mitochondrial injury during torpor and facilitating rapid recruitment of oxidative capacity during
21 arousal.

1 Introduction

2 Hibernating thirteen-lined ground squirrels (*Ictidomys tridecemlineatus* Mitchell), like
3 other small mammalian hibernators, alternate between two distinct metabolic states throughout
4 winter: torpor and interbout euthermia (IBE; Young, 1990; Grahn et al., 1994; Brown et al.,
5 2012). During torpor, metabolic rate (MR) is less than 5% of basal metabolic rate and core body
6 temperature (T_b) is maintained at near-ambient levels (Geiser, 2004; Brown et al., 2012). Torpor
7 bouts are spontaneously interrupted every 7-12 days by periods of IBE, during which T_b returns
8 to $\sim 37^\circ\text{C}$ and MR is maintained at typical resting levels (10-20-fold higher than during torpor)
9 for up to 9 hours. Transitions between torpor and IBE occur very rapidly: arousal from torpor to
10 IBE takes only a few hours, and entrance into torpor from IBE occurs within a single day.

11 A tremendous amount of research has been devoted to elucidating the mechanisms by
12 which hibernating animals can rapidly, reversibly, and repeatedly suppress their metabolism to
13 such a considerable extent. In particular, much attention has been paid to the role of
14 mitochondrial oxidative phosphorylation, as this process is responsible for up to 90% of whole-
15 animal oxygen consumption in mammals (Rolfe and Brown, 1997). A large number of studies,
16 using succinate as a respiratory substrate, have shown that mitochondrial state 3
17 (phosphorylating) respiration rate is reduced up to 83% and 32% during torpor compared to IBE
18 in liver (Gehrich and Aprille, 1988; Muleme et al., 2006; Gerson et al., 2008; Armstrong and
19 Staples, 2010; Chung et al., 2011; Brown et al., 2012) and skeletal muscle (Brown et al., 2012),
20 respectively, when measured *in vitro* at 37°C . When measured at low, torpid-like temperatures
21 (i.e., $4\text{-}10^\circ\text{C}$), lower respiration rates during torpor have been observed in some (Pehowich and
22 Wang, 1984) but not all (Muleme et al., 2006; Brown et al., 2012) studies. Suppression of
23 mitochondrial state 3 respiration during torpor has also been observed with several NADH-
24 linked substrates, including pyruvate, glutamate, and β -hydroxybutyrate, at least in liver
25 (Fedotcheva et al., 1985; Gehrich and Aprille, 1988; Brustovetsky et al., 1990; Muleme et al.,
26 2006). On this basis, it has been proposed that active suppression of mitochondrial oxidative
27 capacity occurs during torpor and may contribute to the reduction of whole-animal metabolism
28 that characterizes mammalian torpor.

29 All previous studies of mitochondrial metabolism during hibernation used only a single,
30 saturating substrate concentration in order to assess differences in maximal oxidative capacity
31 among metabolic states. There are two problems with this approach. First, physiological

1 substrate concentrations are likely less than saturating, so studies using only saturating substrate
2 concentrations cannot address whether mitochondrial suppression may occur under *in vivo*
3 conditions. Second, at the enzyme level, it has been proposed that changes to substrate affinity
4 may play a greater role in regulating enzyme activity at *in vivo* substrate concentrations than
5 changes in maximal enzyme activity (Somero, 1969). If the same is true at the mitochondrial
6 level, then examining only maximal mitochondrial respiration rates may not allow for a complete
7 understanding of the regulation of mitochondrial metabolism during torpor. In particular,
8 alterations in the kinetics of mitochondrial responses to changes in substrate concentration could
9 either dampen or further enhance the effects of reduced oxidative capacity during torpor.
10 Therefore, the first objective of the present study was to measure succinate-fueled respiration at
11 37°C and 10°C in liver and skeletal muscle mitochondria from torpid and IBE hibernators over a
12 wide range of succinate concentrations (from 0.1mM to 10mM).

13 A second objective of the present study was to further examine the role of succinate
14 dehydrogenase (SDH) inhibition in the suppression of succinate-fueled metabolism during
15 torpor. Maximal SDH activity is significantly lower during torpor compared to IBE in liver
16 (Gehrich and Aprille, 1988; Armstrong et al., 2010; Chung et al., 2011), but, to our knowledge,
17 no studies have examined SDH activity in skeletal muscle in any hibernating species. In addition,
18 similar to the studies of mitochondrial respiration, SDH activity has always been measured at
19 37°C using only a single, saturating succinate concentration in order to obtain maximal rates;
20 that is, no studies have examined whether SDH activity is inhibited at torpid-like temperatures or
21 whether there are changes in SDH kinetics during torpor. Therefore, we measured SDH activity
22 in both liver and skeletal muscle at 37°C and 10°C over the same range of succinate
23 concentrations used to assess mitochondrial respiration rates. In addition, we examined whether
24 respiration rates of intact mitochondria correlated with SDH activity measured in the same
25 mitochondria at all succinate concentrations and both assay temperatures.

26 One of the most potent and well-characterized inhibitors of SDH is oxaloacetate (OAA;
27 Wojtczak et al., 1969; Zeylemaker et al., 1969), and a few studies of hibernators have suggested
28 that OAA inhibits SDH activity during torpor (Fedotcheva et al., 1985; Armstrong and Staples,
29 2010; but see also Brustovetsky et al., 1989). Armstrong and Staples (2010) found no significant
30 increase in liver OAA concentration during torpor in thirteen-lined ground squirrels. While this
31 does not preclude the possibility that intramitochondrial OAA levels may increase during torpor,

1 it might also suggest that SDH inhibition during torpor results from increased affinity of SDH for
2 OAA. Therefore, the final objective of the present study was to examine the effects of OAA on
3 liver and skeletal muscle SDH kinetics during torpor and IBE at both 37°C and 10°C.

4 5 **Experimental Procedures**

6 *Animals.* This project conformed to the guidelines of the Canadian Council on Animal
7 Care and was approved by the local Animal Use Subcommittee (protocol 2008-055-06).
8 Thirteen-lined ground squirrels were live-trapped in late May in Carman, MB, Canada (49°30'N,
9 98°01'W). Additionally, some animals were born in captivity to wild-caught pregnant females.
10 Both male and female individuals were used. Animals were housed individually in plastic cages
11 (26.7 x 48.3 x 20.3 cm) and provided with corn-cob bedding, EnviroDri shredded paper bedding
12 (used for nest building), a transparent red polycarbonate tube (for enrichment, 8 x 15 cm; Bio-
13 Serv, Frenchtown, NJ, USA), and a wooden chew stick (to prevent overgrown teeth). They were
14 housed at 22°C ± 3°C with photoperiod adjusted weekly to match that of Carman, MB. Rodent
15 chow (Lab Diet 5P00), dry dog food (Iams), and tap water were provided *ad libitum*, with
16 sunflower seeds, dry corn cobs, carrots, and mealworms provided periodically, especially to
17 post-weaned young-of-the-year. Bedding materials and rodent chow were purchased from Ren's
18 Pets Depot (Oakville, ON, Canada), whereas all other animal supplies were obtained from local
19 pet shops and grocery stores.

20 In July, radiotelemeters (TA-F10; Data Sciences International, St. Paul, MN, USA) were
21 implanted into the intraperitoneal space under isofluorane anaesthesia to allow for T_b monitoring
22 (Muleme et al. 2006). Ketoprofen (Anafen; Merial Canada Inc., Baie d'Urfé, QC, Canada; 10
23 mg/mL, 0.1 mL/100g) was provided as an analgesic prior to, and for three days following,
24 surgery. In October, animals began to hibernate and were moved to an environmental chamber
25 maintained at 4°C ± 2°C on a 2:22-h light:dark photoperiod (lights on at 8:00 EST). After 1
26 week of uninterrupted torpor, food (but not water) was withheld. All animals were sampled
27 throughout winter (from December to March) at the same time of day (8:00-10:00 EST). Torpid
28 animals were sampled when T_b was at or below 5°C for at least 3 days and were euthanized via
29 cervical dislocation. IBE animals were sampled following spontaneous arousal from torpor when
30 T_b was at or above 36°C for at least one hour and were euthanized via anaesthetic overdose
31 (Euthanyl; Bimeda-MTC, Cambridge, ON, Canada; 270 mg/mL, 0.2 mL/100 g). These different

1 euthanasia methods are required by our animal care protocols, but Euthanyl has no effects on
2 mitochondrial metabolism (Takaki et al., 1997).

3 *Mitochondrial isolation and respiration rate.* All chemicals used for mitochondrial
4 isolation and subsequent measurements were obtained from Sigma-Aldrich (Oakville, ON,
5 Canada). Purified liver and skeletal muscle mitochondria were isolated via differential
6 centrifugation followed by density-gradient centrifugation, as described previously (Brown et al.,
7 2012). Mitochondrial respiration rates were determined using a high-resolution Oxygraph
8 respirometer (Oroboros; Innsbruck, Austria). Oxygen electrodes were calibrated to air-saturated
9 buffer at both 37°C and 10°C using published oxygen solubilities (Forstner and Gnaiger, 1983),
10 corrected for local atmospheric pressure.

11 Succinate-fueled respiration was measured by suspending liver (~100 µg protein) or
12 skeletal muscle (~30 µg protein) mitochondria in 2.5 mL of MiR05 assay buffer (Gnaiger and
13 Kuznetsov, 2002) equilibrated to either 37°C or 10°C. We chose to use 10°C, a temperature
14 slightly above torpid T_b (approximately 5°C), because, in our experience, it is the lowest *in vitro*
15 temperature that yields reliable respiration measurements. Rotenone (2 µg/mL, dissolved in
16 ethanol) was added to inhibit complex I of the mitochondrial electron transport chain and prevent
17 reverse electron flow. Succinate (0.1 – 10 mM) was added to stimulate state 2 respiration, and,
18 subsequently, ADP (0.2 mM) was added to stimulate state 3 respiration. Once a steady-state rate
19 had been achieved, oligomycin (2 µg/mL, dissolved in ethanol; inhibitor of F_1F_0 -ATPase) was
20 added to approximate state 4 conditions.

21 *Succinate dehydrogenase activity and oxaloacetate affinity.* For measurements of SDH
22 activity, mitochondria (sonicated on ice 2 x 10 sec. with 30 seconds between; ~10 µg protein
23 liver and ~3 µg protein skeletal muscle; or buffer alone for blanks) were added to 200 µL of
24 SDH buffer (55 mM KH_2PO_4 , 25 mM sucrose, and 0.11% w/v idonitrotetrazolium chloride, pH
25 7.4 at respective assay temperature, either 37°C or 10°C). Subsequently, succinate (0.05 – 10
26 mM) was added to stimulate SDH activity. Absorbance (500 nm) was measured every 15
27 seconds for 10 minutes using a spectrophotometer (SpectraMax M2^e; Molecular Devices,
28 Sunnyvale, CA, USA), and initial linear rates were used to calculate kinetic parameters.

29 OAA acts as a competitive inhibitor when SDH is pre-incubated with succinate, and a
30 mixed inhibitor without succinate pre-incubation (Mandrik et al., 1983). There is no evidence
31 that suppression of mitochondrial succinate oxidation during torpor occurs via competitive

1 inhibition, as it occurs even at saturating succinate levels, so we chose to examine OAA
2 inhibition of SDH by pre-incubating the enzyme with OAA in the absence of succinate.
3 Mitochondria (concentrations as above) were added to 200 μ L of SDH buffer and pre-incubated
4 for 5 minutes with 5 μ M OAA. This concentration of OAA was selected because it approximates
5 the difference in tissue OAA concentration observed between torpor and IBE (Armstrong et al.,
6 2010). Following pre-incubation, succinate was added to stimulate SDH activity and absorbance
7 was measured as described above.

8 *Data analysis.* Values presented are means \pm s.e.m, except where $N = 2$, in which cases
9 the range is shown. To examine differences in mitochondrial respiration rate and SDH activity
10 between metabolic states over the range of succinate concentrations, mitochondrial respiration
11 and enzyme activity data for each tissue and assay temperature were analyzed separately using a
12 linear mixed model analysis with repeated measures (succinate concentration). To examine
13 differences in enzyme kinetics between metabolic states, maximal enzyme activity (V_{\max}) and
14 apparent Michaelis-Menten constant ($\text{App}K_M$) were estimated using SigmaPlot 2001 (SPSS Inc.,
15 Chicago, Illinois, USA) by fitting the mitochondrial respiration or SDH activity data to the
16 Michaelis-Menten equation (single rectangular hyperbola). Inhibition constants (K_i and K_i') for
17 OAA were determined by calculating V_{\max} and $\text{App}K_M$ in the absence and presence of OAA and
18 solving the extended Michaelis-Menten equation for these parameters. Subsequently, kinetic
19 parameters for each tissue were analyzed separately using a linear mixed model with repeated
20 measures (temperature). In all analyses, non-significant interactions were removed from the
21 model. All statistical analyses were conducted using SPSS version 19.0 (SPSS Inc, Chicago,
22 Illinois, USA).

23 24 **Results**

25 *Respiration rate is lower in mitochondria from torpid animals regardless of succinate*
26 *concentration or assay temperature.* When measured at 37°C, state 3 respiration rate of both
27 liver (Fig. 1A) and skeletal muscle (Fig. 1B) mitochondria was significantly lower in torpid
28 animals, regardless of succinate concentration used, with the exception of 0.1 mM succinate in
29 liver. With saturating (10 mM) succinate levels, the extent of suppression (52% and 33% in liver
30 and skeletal muscle, respectively, compared with IBE) was comparable to previous studies
31 (Gehrich and Aprille, 1988; Muleme et al., 2006; Gerson et al., 2008; Armstrong and Staples,

1 2010; Chung et al., 2011; Brown et al., 2012), but the extent of suppression tended to decline
2 with succinate concentration. For example, with 1 mM succinate, the extent of suppression was
3 42% in liver and 12.5% in skeletal muscle. Similar results were obtained when mitochondrial
4 respiration rate was measured at 10°C. In liver (Fig. 1C), state 3 respiration rate was significantly
5 lower (50-60%) in torpid animals down to 1 mM succinate, while at lower succinate levels, no
6 difference was observed. In skeletal muscle (Fig. 1D), state 3 respiration was suppressed at all
7 succinate concentrations, but the extent of suppression was greater at saturating levels (e.g.,
8 51.5% at 10 mM) compared to sub-saturating levels (e.g., 39% at 1 mM).

9 *Succinate dehydrogenase activity is lower in torpor at 37°C—but not 10°C—regardless*
10 *of succinate concentration.* We measured SDH activity at the same succinate concentrations and
11 assay temperatures used to measure mitochondrial respiration. In order to better resolve SDH
12 kinetics, we additionally measured SDH activity with 0.25 mM succinate at 37°C and 0.05 mM
13 succinate at 10°C. At 37°C, SDH activity was significantly lower in torpid animals compared
14 with IBE in both liver (Fig. 2A) and skeletal muscle (Fig. 2B) regardless of succinate
15 concentration, again with the exception of liver at 0.1 mM succinate. As with mitochondrial
16 respiration, the extent of inhibition of SDH tended to be higher at saturating succinate levels. In
17 liver, SDH was 38% lower at 10 mM succinate but only 30% lower at 1 mM, and, in skeletal
18 muscle, SDH was 24% lower at 10 mM succinate but only 18% lower at 1 mM. At 10°C, on the
19 other hand, no difference in SDH activity between metabolic states was observed in either liver
20 (Fig. 2C) or skeletal muscle (Fig. 2D) at any succinate concentration.

21 The extent of SDH inhibition during torpor correlated well with differences in the extent
22 of suppression of mitochondrial succinate oxidation both between and within tissues when
23 measured at 37°C. The extent of both SDH inhibition and mitochondrial succinate oxidation
24 suppression were higher in liver compared with skeletal muscle (compare data in Figs. 1 and 2).
25 Moreover, within both liver and skeletal muscle, there was a strong correlation between SDH
26 activity and mitochondrial succinate oxidation rate at all succinate concentrations where
27 suppression of mitochondrial respiration was observed, with the notable exception of 0.5 and 0.1
28 mM in skeletal muscle (Fig. 3); however, the strength of the correlation tended to decline at
29 lower succinate levels in both tissues.

30 *Kinetics of mitochondrial respiration and SDH are altered during torpor, especially in*
31 *liver.* The kinetics of both mitochondrial respiration (Fig. 4A) and SDH (Fig. 4B) were assessed

1 by fitting data from Figs. 1 and 2 to the Michaelis-Menten equation. This equation generally fit
2 the data quite well ($r^2 > 0.9$), except for respiration data measured at 10°C ($r^2 = 0.49-0.85$
3 depending on tissue and metabolic state). While this may reduce the accuracy of our estimates of
4 $AppK_M$ values at 10°C for the mitochondrial respiration data, we believe that these values reflect
5 the clear trends in the data. In both tissues and at both assay temperatures, the extent of decline in
6 mitochondrial respiration rate at lower succinate concentrations was reduced in torpor. For
7 example, for liver mitochondrial respiration measured at 37°C, the respiration rate measured
8 using 1mM succinate was 48% lower compared to that measured using 10mM succinate for IBE
9 mitochondria; however, the reduction was only 38% for torpid mitochondria in torpor over the
10 same change in concentration. Similar findings were observed in skeletal muscle at 37°C, and
11 both tissues at 10°C. Therefore, for liver mitochondrial respiration, we observed that $AppK_M$ was
12 significantly lower in torpor than IBE at both assay temperatures. For skeletal muscle respiration,
13 similar trends were seen, especially at 37°C, but they did not reach statistical significance.

14 In both liver and skeletal muscle, $AppK_M$ values were considerably (~5-fold) lower for
15 SDH than mitochondrial respiration. In liver, similar to mitochondrial respiration, $AppK_M$ was
16 significantly lower in torpor than IBE at both 37°C and 10°C. In skeletal muscle, by contrast, as
17 with mitochondrial respiration, $AppK_M$ values showed no significant difference between
18 metabolic states for either mitochondrial respiration or SDH activity, though they tended to be
19 lower in torpor at 37°C.

20 *SDH from torpid animals has a greater apparent affinity for oxaloacetate.* We also
21 measured SDH kinetics following a 5-minute pre-incubation with OAA in both liver (Fig. 5A)
22 and skeletal muscle (Fig. 5B). Pre-incubation with OAA in the absence of succinate allowed
23 OAA to act as a mixed inhibitor of SDH (Mandrik et al., 1983). Under these conditions, OAA
24 can bind to SDH when succinate is not bound but also when succinate is already bound, although
25 with different affinities, termed K_i and K_i' , respectively. We observed that OAA had a much
26 lesser apparent affinity for SDH when succinate was previously bound than when it was not
27 already bound (K_i' was 15-30-fold higher than K_i), and that K_i' did not differ between metabolic
28 states in either tissue (data not shown). However, K_i was significantly lower in torpor in both
29 liver and skeletal muscle at 37°C and 10°C (Fig. 5C), suggesting that the affinity of SDH for
30 OAA is greater in torpor, regardless of assay temperature or tissue.

31

1 Discussion

2 *In torpor, mitochondria exhibit reduced oxidative capacity over a range succinate*
3 *concentrations but are less responsive to changes in substrate concentration.* The present study
4 demonstrates that suppression of succinate-fueled respiration occurs with both saturating and
5 sub-saturating succinate concentrations. This observation is important because sub-saturating
6 succinate concentrations likely better reflect physiological conditions. While, to our knowledge,
7 there are no studies of intramitochondrial substrate levels during *in vivo* metabolism, reports of
8 tissue succinate concentrations range from < 0.1 mM in mouse liver and skeletal muscle (Lin et
9 al. 2011) to <0.5 mM in rabbit heart (Lewandowski et al., 1996) to ~1 mM in liver from rats
10 (Busch and Potter, 1952) and hibernating ground squirrels (Serkova et al., 2007). Bollard et al.
11 (2003) suggest that succinate is largely a mitochondrial metabolite, being more readily detected
12 in mitochondrial extracts than whole tissue extracts using NMR-based techniques; therefore,
13 concentrations reported for whole tissue likely underestimate intramitochondrial levels.
14 Notwithstanding, we are confident that the range of succinate concentrations used in this study
15 spans the physiological range. Thus, the present study demonstrates that mitochondrial oxidative
16 capacity is likely reduced during torpor *in vivo* and can play an important role in reducing MR.

17 The present study also demonstrates that mitochondria are less responsive to changes in
18 substrate concentration in torpor than in IBE. In liver, this fact is reflected by the lower AppK_M
19 value for succinate-fueled respiration in torpor, and a similar trend was observed in skeletal
20 muscle, at least at 37°C. This lower sensitivity to changing substrate levels in torpor means that
21 current estimates of energy savings achieved through mitochondrial suppression are likely
22 overestimates because they are based on measurements made using saturating succinate
23 concentrations where the difference in oxygen consumption rates between IBE and torpor are
24 maximal. At sub-saturating substrate concentrations, likely more representative of physiological
25 conditions, the difference between IBE and torpor is smaller. Therefore, while hibernator
26 mitochondria are clearly modified to reduce their capacity to oxidize substrates during torpor,
27 they appear to be simultaneously modified to ensure that mitochondrial respiration does not
28 decline to very low levels during torpor but rather remains above some critical threshold. These
29 modifications may allow for both energy savings during torpor as well as the rapid restoration of
30 high mitochondrial oxidation rates during arousal. In addition, maintaining a minimal level of
31 substrate oxidation during torpor may prevent mitochondrial membrane depolarization (and,

1 therefore, apoptosis; Ly et al., 2003). Indeed, we have previously shown that mitochondrial
2 membrane potential remains fairly constant during torpor in ground squirrels (Brown et al.,
3 2012), even at 10°C.

4 The kinetics of mitochondrial respiration should reflect the underlying kinetics of those
5 components of mitochondrial metabolism that bind succinate prior to its oxidation, in particular,
6 the dicarboxylate transporter (DCT) and SDH. In liver, we observed that SDH had a greater
7 apparent affinity for succinate during torpor than IBE regardless of assay temperature. A similar
8 trend was observed in skeletal muscle at 37°C, but it did not reach statistical significance.
9 Therefore, increased affinity of SDH for succinate may contribute to the blunted responsiveness
10 of mitochondrial respiration to declining succinate concentrations in torpor. The kinetics of other
11 metabolic enzymes have also been reported to change during torpor. D-3-hydroxybutyrate
12 dehydrogenase generally had a greater affinity for several substrates in hibernating compared to
13 active jerboas when measured at 37°C (Kabine et al., 2003). Thatcher and Storey (2001)
14 reported that K_M for glutamate dehydrogenase (GDH) from liver of torpor Richardson's ground
15 squirrels was lower compared to GDH from euthermic animals, but only when measured at 5°C.
16 At 37°C, the opposite pattern was observed, suggesting that GDH in hibernating animals is
17 optimized for substrate binding at the prevailing T_b . Moon (1978) reported similar results for
18 lactate dehydrogenase in a number of tissues in hibernating bats, where affinity for pyruvate was
19 greater in torpid individuals, particularly at temperatures below 37°C. Therefore, it appears that
20 enzymes from hibernating animals generally have a greater affinity for their substrates,
21 especially at torpid-like temperatures.

22 While changes to SDH kinetics may contribute to changes to mitochondrial respiration
23 kinetics, the $AppK_M$ values measured for SDH were 5-fold lower than those values for
24 mitochondrial respiration. Moreover, there appears to be a better correspondence between our
25 $AppK_M$ values for mitochondrial respiration and those measured previously for succinate kinetics
26 of DCT ($K_M = 1.2$ mM; Palmieri et al., 1971). Therefore, it seems likely that the kinetics of
27 mitochondrial respiration largely reflect those of succinate transport, and we predict that the
28 DCT may have a greater affinity for succinate in torpid animals. To our knowledge, there is
29 presently little information about how mitochondrial substrate transport is regulated during
30 torpor, though the data in the present study suggest this merits further consideration.

1 *SDH inhibition may initiate—but does not maintain—suppression of succinate-fueled*
2 *metabolism during torpor.* How mitochondrial metabolism is suppressed during torpor remains
3 to be fully elucidated, but a number of mechanisms have been proposed (Shug et al., 1971;
4 Lerner et al., 1972; Fedotcheva et al., 1985; Brustovetsky et al., 1989; Brustovetsky et al., 1990;
5 Barger et al., 2003; Armstrong et al., 2010; Brown et al., 2012). With regards to succinate-fueled
6 metabolism, our finding of lower SDH maximal activity (measured at 37°C) in liver during
7 torpor is consistent with previous studies (Gehrich and Aprille, 1988; Armstrong and Staples,
8 2010; Chung et al., 2011). For the first time, however, we also report lower maximal SDH
9 activity in skeletal muscle during torpor. SDH inhibition in torpor was also evident at sub-
10 saturating succinate concentrations. In liver, SDH activity correlated well with succinate-fueled
11 mitochondrial respiration rate at all concentrations where SDH inhibition and mitochondrial
12 metabolic suppression were observed. Thus, SDH inhibition likely plays an important role in the
13 active suppression of liver mitochondrial metabolism *in vivo*. In skeletal muscle, on the other
14 hand, while SDH activity accounted for up to 55% of the variation in mitochondrial respiration at
15 ≥ 1 mM succinate, there was no relationship between SDH activity and mitochondrial respiration
16 at either 0.1 mM or 0.5 mM despite that both parameters were inhibited in torpid animals at these
17 concentrations. This observation suggests that, at low substrate levels, SDH has little control
18 over mitochondrial respiration in skeletal muscle. Our data do not permit us to assess how
19 control of respiration is redistributed among the components of oxidative phosphorylation at low
20 succinate levels in this tissue, but the DCT has been shown to exhibit considerable control over
21 succinate-fueled respiration at low succinate levels (Quagliariello and Palmieri, 1968) and may
22 contribute to mitochondrial suppression in this tissue *in vivo*. It is known that the distribution of
23 control over mitochondrial respiration varies among tissues (Rossignol et al., 2000); therefore, it
24 seems reasonable that the mechanisms that bring about suppression of succinate oxidation during
25 torpor may vary among tissues accordingly.

26 Unlike at 37°C, SDH activity did not differ between torpor and IBE when measured at
27 10°C in either liver or skeletal muscle despite that mitochondrial respiration was suppressed in
28 both tissues at this low temperature. Therefore, SDH inhibition likely only contributes to the
29 initiation of mitochondrial metabolic suppression during entrance into torpor, when T_b is still
30 high. Indeed, the maximal extent of active mitochondrial suppression occurs very early during
31 entrance into torpor, before T_b falls to 30°C (Chung et al., 2011). At the same time, some

1 mechanism other than SDH must maintain mitochondrial metabolic suppression during torpor,
2 when T_b is low. Two previous studies (Fedotcheva et al., 1985; Armstrong and Staples, 2010)
3 suggested that OAA binding inhibits SDH activity during torpor, but tissue OAA levels do not
4 change among hibernation metabolic states in ground squirrel liver (Armstrong and Staples,
5 2010). While tissue levels do not necessarily reflect intramitochondrial OAA levels, this
6 observation suggests that increased affinity of SDH for OAA in torpor could be the mechanism
7 of SDH inhibition. Indeed, we found that the affinity of SDH for OAA is much greater during
8 torpor in both liver and skeletal muscle regardless of assay temperature despite that SDH was not
9 inhibited at 10°C. This observation strengthens the contention that OAA plays a role in SDH
10 inhibition during torpor but also suggests that the lack of SDH inhibition at 10°C does not result
11 from the loss of bound OAA. It may be, instead, that SDH is less temperature-sensitive during
12 torpor than IBE.

13 *Perspectives and significance.* The present study examined mitochondrial function in
14 hibernating animals over a wide range of succinate concentrations that almost undoubtedly span
15 the physiological succinate range. We chose to examine succinate-fueled metabolism in the
16 present study because i) it is the most common substrate used in studies of mitochondrial
17 metabolism in hibernating animals, and ii) it allowed us to examine changes to an important
18 segment of the electron transport chain without confounding effects from upstream processes
19 that may be differentially regulated, for example, to shift fuel use away from carbohydrates
20 rather than to suppress metabolism *per se* (Buck et al., 2002). For the most part, the results
21 confirmed observations in previous studies made using saturating succinate concentrations
22 (Gehrich and Aprille, 1988; Muleme et al., 2006; Gerson et al., 2008; Armstrong and Staples,
23 2010; Chung et al., 2011; Brown et al., 2012), but two important facts were revealed by the
24 present study. First, the degree of mitochondrial metabolic suppression during torpor is lower at
25 sub-saturating compared to saturating succinate levels because torpid mitochondria are less
26 sensitive to changes in succinate concentration than IBE mitochondria. Therefore, studies using
27 saturating substrate levels may have overestimated the extent of mitochondrial metabolic
28 suppression (and related energy savings) during torpor. Second, in skeletal muscle, although both
29 succinate-fueled respiration and SDH activity are reduced in torpor at all substrate
30 concentrations, there was no correlation between the two parameters at succinate concentrations
31 < 1 mM. This suggests that SDH may have little control over respiration in skeletal muscle at

1 physiological succinate concentrations and, therefore, may not play a significant role in
2 suppressing mitochondrial metabolism during torpor in this tissue. This is not the conclusion that
3 would be drawn using only saturating substrate concentrations. In addition, mitochondria in
4 hibernating animals function over a range of temperatures, and, therefore, mitochondrial
5 metabolism should be assessed at several representative temperatures. Many previous studies of
6 mitochondrial metabolism in hibernating animals examined mitochondrial function at non-
7 physiological temperatures (esp. $\sim 25^{\circ}\text{C}$; Martin et al., 1999; Fedotcheva et al., 1985;
8 Brustovetsky et al., 1989) or at euthermic temperatures only (37°C ; e.g., Barger et al., 2003;
9 Armstrong and Staples, 2010), and extrapolated their results to the entire hibernation season. The
10 present study clearly highlights the shortcoming of this approach. When measured at torpid-like
11 temperatures, SDH activity was not reduced in torpor, and SDH inhibition likely plays little role
12 in maintaining metabolic suppression during torpor when T_b is low. All of these nuances of
13 mitochondrial metabolic regulation in hibernating animals would have been overlooked had we
14 used only a single, saturating succinate concentration at a single assay temperature.

15 The results of the present study also highlight two important research directions. First, the
16 mechanisms by which SDH is altered during torpor remain to be elucidated. Recently, Phillips et
17 al. (2012) contended that mitochondrial oxidative phosphorylation complexes are acutely
18 regulated via post-translational modifications. The phosphorylation state of SDH does not
19 change throughout the hibernation season (D.J. Chung, J.C.L. Brown, B. Syzszka, N.P.A. Huner,
20 and J.F. Staples, unpublished), but SDH could be modulated via other post-translational
21 modifications, especially acetylation (Cimen et al. 2010). Certainly, some proteins are well-
22 known to be post-translationally modified during hibernation, for example, phosphorylation of
23 pyruvate dehydrogenase (Andrews et al. 1998) and glutamate dehydrogenase (Bell and Storey
24 2010), as well as SUMOylation of several transcription factors (Lee et al. 2007). Second,
25 mechanisms other than SDH inhibition are likely to influence the initiation and maintenance of
26 mitochondrial succinate oxidation during torpor. These are most likely other components of the
27 substrate oxidation subsystem (i.e., substrate transporters or other ETC complexes), as previous
28 work (Brown et al. 2012) has provided little evidence for a role for any component of the ADP
29 phosphorylation subsystem (i.e., ATP synthase, phosphate and adenine nucleotide transporters).
30 One site that we believe is worth further examination is the dicarboxylate transporter. In fact, we

1 generally believe that more emphasis should be placed on the potential role of substrate
2 transporters in regulating mitochondrial metabolism during torpor in hibernating mammals.

3
4

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14

15 **Symbols and Abbreviations Used**

16 MR, metabolic rate; T_b , body temperature; IBE, interbout euthermia; SDH, succinate
17 dehydrogenase; OAA, oxaloacetate; V_{max} , maximal enzyme activity; AppKM, apparent
18 Michaelis-Menten constant; K_i , inhibition constant; $\Delta\psi_m$, mitochondrial membrane potential;
19 DCT, dicarboxylate transporter; GDH, glutamate dehydrogenase.

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1 **Figure Captions.**

2

3 **Fig.1. Mitochondrial respiration rates measured over a range of succinate concentrations.**

4 Respiration rates of mitochondria isolated during interbout euthermia (open circles) and torpor
5 (filled circles) were measured in liver and skeletal muscle at both 37°C and 10°C using six
6 different concentrations of succinate in the presence of rotenone. P-values for main effects and
7 interactions are shown in each panel. Where the interaction effect was significant, asterisks (*)
8 indicate significant ($P < 0.05$) differences between metabolic states at each succinate
9 concentration. Sample size for each metabolic state is indicated in parentheses.

10

11 **Fig. 2. Succinate dehydrogenase (SDH) activity measured over a range of succinate**

12 **concentrations.** SDH activity of interbout euthermic (open circles) and torpid (filled circles)
13 animals were measured in liver and skeletal muscle at both 37°C and 10°C using seven different
14 concentrations of succinate. P-values for main effects and interactions are shown in each panel.
15 Where the interaction effect was significant, asterisks (*) indicate significant ($P < 0.05$)
16 differences between metabolic states at each succinate concentration. Sample size for each
17 metabolic state is indicated in parentheses.

18

19 **Fig. 3. Correlation between succinate-fueled state 3 mitochondrial respiration and SDH**

20 **activity measured at 37°C over a range of succinate concentrations for both liver (filled**
21 **circles) and skeletal muscle (open circles).** Significant correlations at each succinate
22 concentration are indicated by symbols. ** $P < 0.01$, * $P < 0.05$. $N = 14$ and 10 for liver and
23 skeletal muscle, respectively, at all succinate concentrations. No correlations are shown for 10°C
24 because SDH activity was not significantly lower during torpor in either tissue at this assay
25 temperature.

26

27 **Fig. 4. Kinetics of mitochondrial respiration and succinate dehydrogenase (SDH).** Apparent

28 Michaelis-Menten constants ($AppK_M$) for succinate were calculated for torpid (filled bars) and
29 interbout euthermic animals (open bars) from mitochondrial respiration rates (A) and SDH
30 activity (B) for both liver and skeletal muscle at 37°C and 10°C. ** $P < 0.01$, * $P < 0.05$ vs.
31 interbout euthermia in the same tissue and at the same temperature. ‡ $P < 0.05$ vs. the same

1 metabolic state and tissue measured at 37°C (i.e., significant effect of temperature). Sample size
2 is indicated in parentheses below each bar.

3

4 **Fig. 5. Effect of oxaloacetate (OAA) on succinate dehydrogenase (SDH) kinetics.** OAA is a
5 mixed inhibitor when pre-incubated without succinate and, therefore, can bind to SDH when it is
6 not already bound to succinate (inhibition constant, K_i) and when it is already bound to succinate
7 (inhibition constant, K_i'). K_i' values did not differ between metabolic states, and so only K_i
8 values are shown for torpid (filled bars) and interbout euthermic (open bars) animals. There was
9 no significant interaction between metabolic state and temperature in either tissue. * $P < 0.05$ vs.
10 interbout euthermia in the same tissue and at the same temperature. ‡ $P < 0.05$ vs. the same
11 metabolic state and tissue measured at 37°C (i.e., significant effect of temperature). Sample size
12 is indicated in parentheses below the corresponding each bar.









