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

Short title: Mitochondrial regulation in hibernation.

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Summary

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

Introduction

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

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

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

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

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

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

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

Experimental Procedures

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

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

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

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

Succinate-fueled respiration was measured by suspending liver (~100 μg protein) or skeletal muscle (~30 μg protein) mitochondria in 2.5 mL of MiR05 assay buffer (Gnaiger and Kuznetsov, 2002) equilibrated to either 37°C or 10°C. We chose to use 10°C, a temperature slightly above torpid T_b (approximately 5°C), because, in our experience, it is the lowest *in vitro* temperature that yields reliable respiration measurements. Rotenone (2 μg/mL, dissolved in ethanol) was added to inhibit complex I of the mitochondrial electron transport chain and prevent reverse electron flow. Succinate (0.1 – 10 mM) was added to stimulate state 2 respiration, and, subsequently, ADP (0.2 mM) was added to stimulate state 3 respiration. Once a steady-state rate had been achieved, oligomycin (2 μg/mL, dissolved in ethanol; inhibitor of F_1F_O-ATPase) was added to approximate state 4 conditions.

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

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

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

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

Results

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

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

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

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

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

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

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

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

Discussion

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

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

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

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

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

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

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

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

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

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

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

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

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Symbols and Abbreviations Used

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

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Figure Captions.

Fig.1. Mitochondrial respiration rates measured over a range of succinate concentrations. Respiration rates of mitochondria isolated during interbout euthermia (open circles) and torpor (filled circles) were measured in liver and skeletal muscle at both 37°C and 10°C using six different concentrations of succinate in the presence of rotenone. P-values for main effects and interactions are shown in each panel. Where the interaction effect was significant, asterisks (*) indicate significant ($P < 0.05$) differences between metabolic states at each succinate concentration. Sample size for each metabolic state is indicated in parentheses.

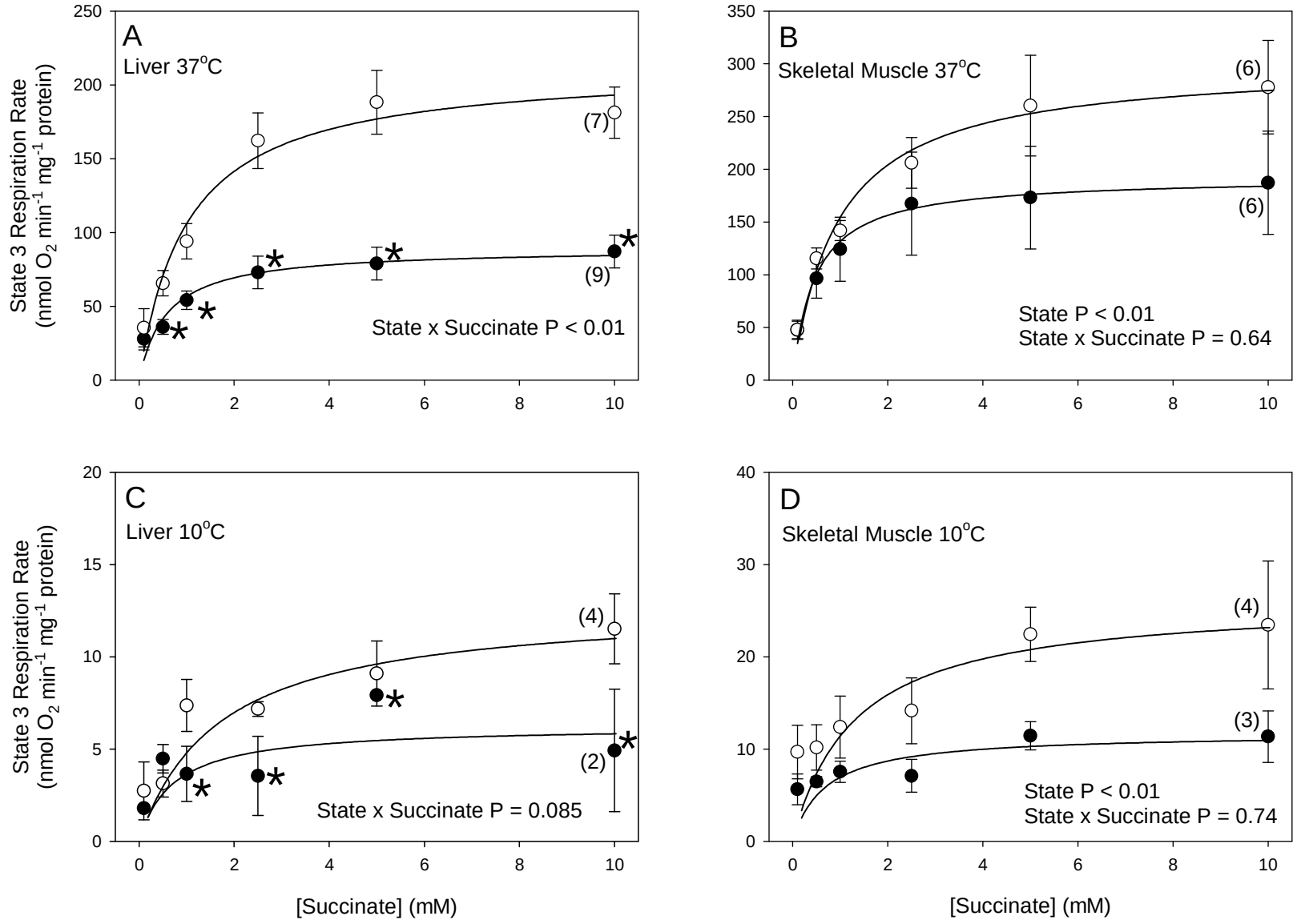
Fig. 2. Succinate dehydrogenase (SDH) activity measured over a range of succinate concentrations. SDH activity of interbout euthermic (open circles) and torpid (filled circles) animals were measured in liver and skeletal muscle at both 37°C and 10°C using seven different concentrations of succinate. P-values for main effects and interactions are shown in each panel. Where the interaction effect was significant, asterisks (*) indicate significant ($P < 0.05$) differences between metabolic states at each succinate concentration. Sample size for each metabolic state is indicated in parentheses.

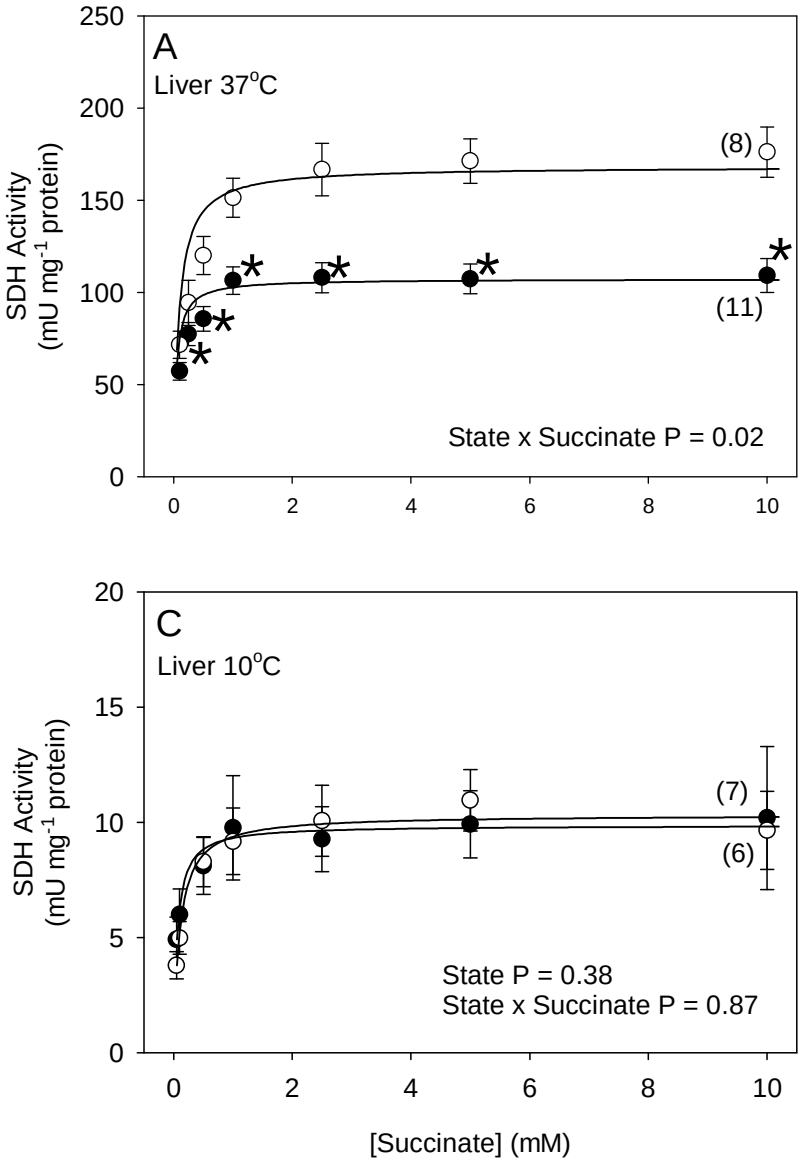
Fig. 3. Correlation between succinate-fueled state 3 mitochondrial respiration and SDH activity measured at 37°C over a range of succinate concentrations for both liver (filled circles) and skeletal muscle (open circles). Significant correlations at each succinate concentration are indicated by symbols. ** $P < 0.01$, * $P < 0.05$. N = 14 and 10 for liver and skeletal muscle, respectively, at all succinate concentrations. No correlations are shown for 10°C because SDH activity was not significantly lower during torpor in either tissue at this assay temperature.

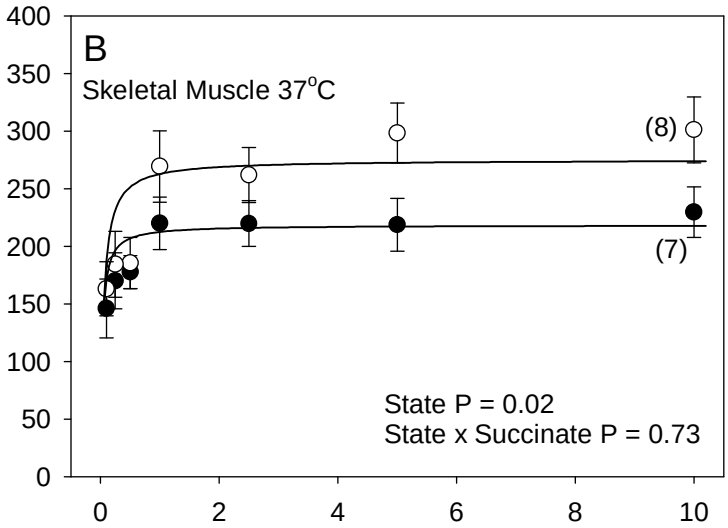
Fig. 4. Kinetics of mitochondrial respiration and succinate dehydrogenase (SDH). Apparent Michaelis-Menten constants ($AppK_M$) for succinate were calculated for torpid (filled bars) and interbout euthermic animals (open bars) from mitochondrial respiration rates (A) and SDH activity (B) for both liver and skeletal muscle at 37°C and 10°C. ** $P < 0.01$, * $P < 0.05$ vs. interbout euthermia in the same tissue and at the same temperature. ‡ $P < 0.05$ vs. the same

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

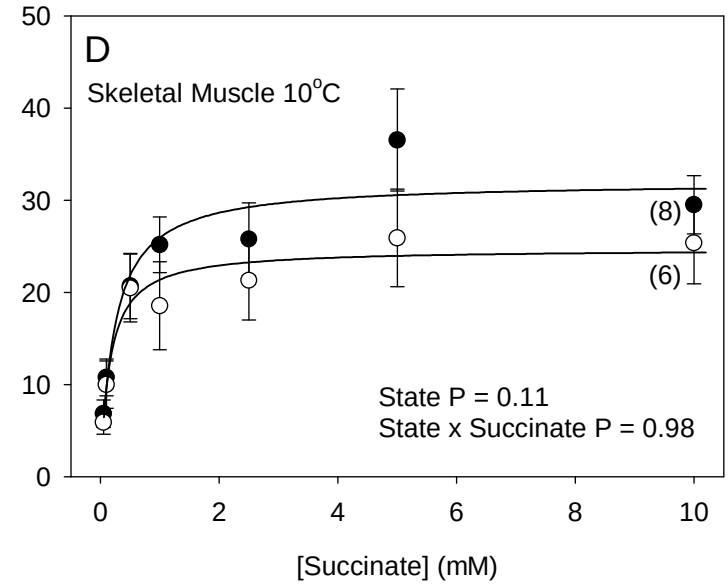
Fig. 5. Effect of oxaloacetate (OAA) on succinate dehydrogenase (SDH) kinetics. OAA is a mixed inhibitor when pre-incubated without succinate and, therefore, can bind to SDH when it is not already bound to succinate (inhibition constant, K_i) and when it is already bound to succinate (inhibition constant,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

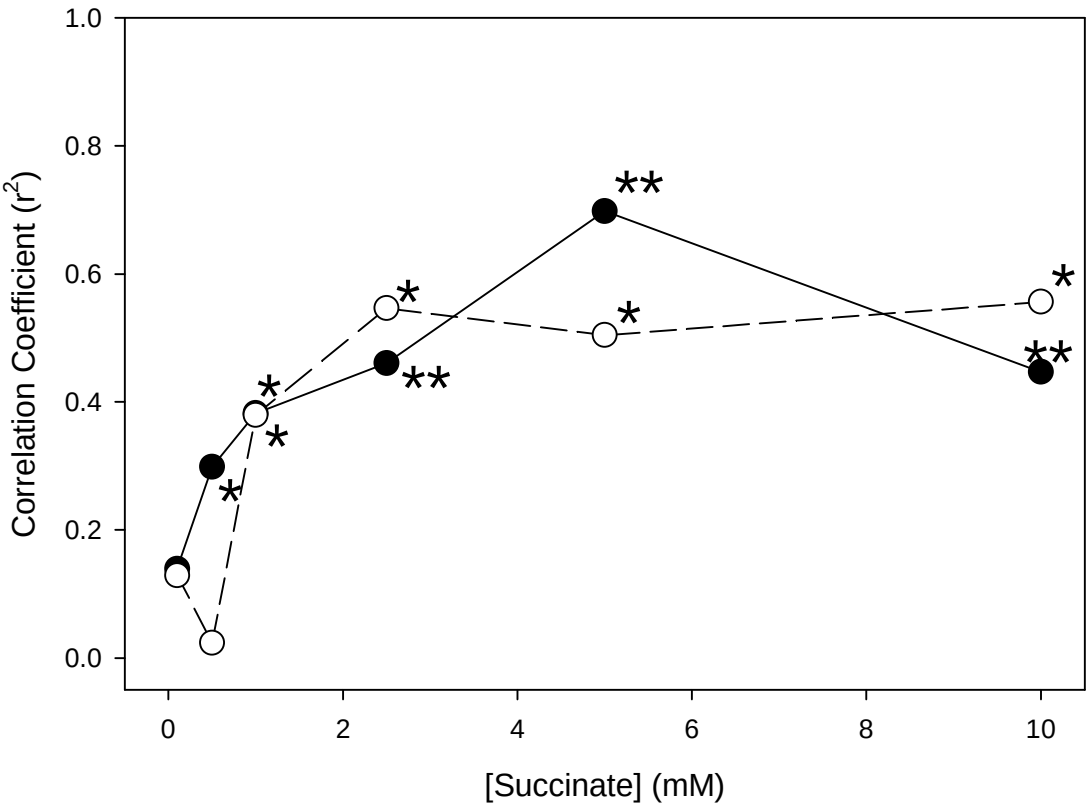

A
Liver 37°C
(7)
(9)
State x Succinate P < 0.01
B
Skeletal Muscle 37°C
(6)
(6)
State P < 0.01
State x Succinate P = 0.64
C
Liver 10°C
(4)
(2)
State x Succinate P = 0.085
D
Skeletal Muscle 10°C
(4)
(3)
State P < 0.01
State x Succinate P = 0.74
State 3 Respiration Rate
(nmol O2 min-1 mg-1 protein)
[Succinate] (mM)

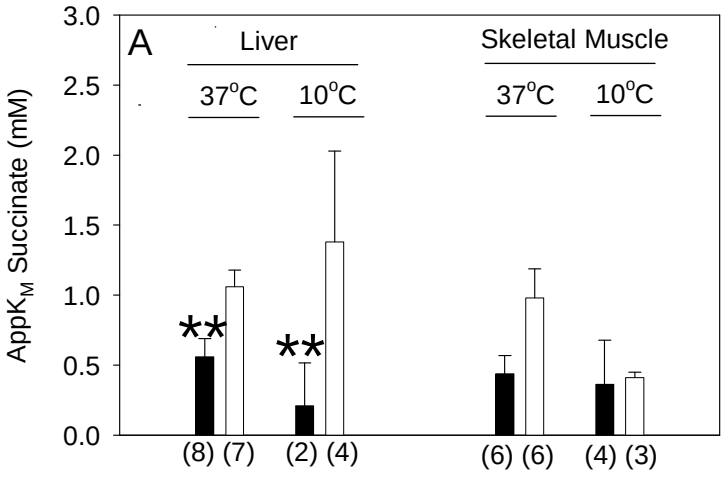

A
Liver 37°C
SDH Activity
(mU mg-1 protein)
(8)
(11)
State x Succinate P = 0.02
0
50
100
150
200
250
0
2
4
6
8
10

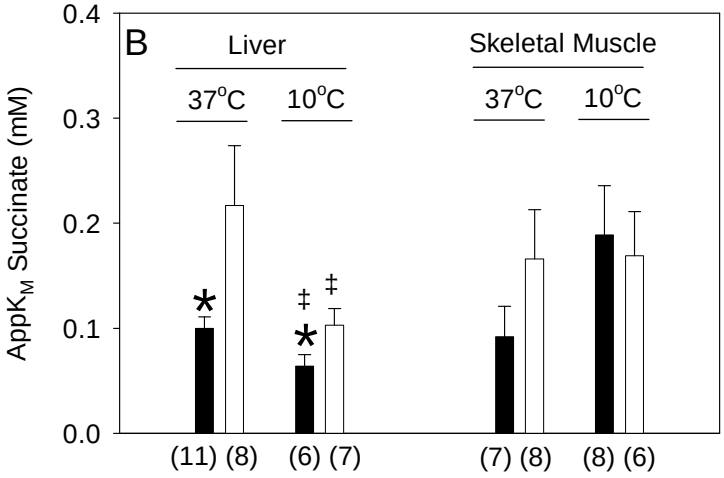

B
Skeletal Muscle 37°C
(8)
(7)
State P = 0.02
State x Succinate P = 0.73
0
50
100
150
200
250
300
350
400
0
2
4
6
8
10

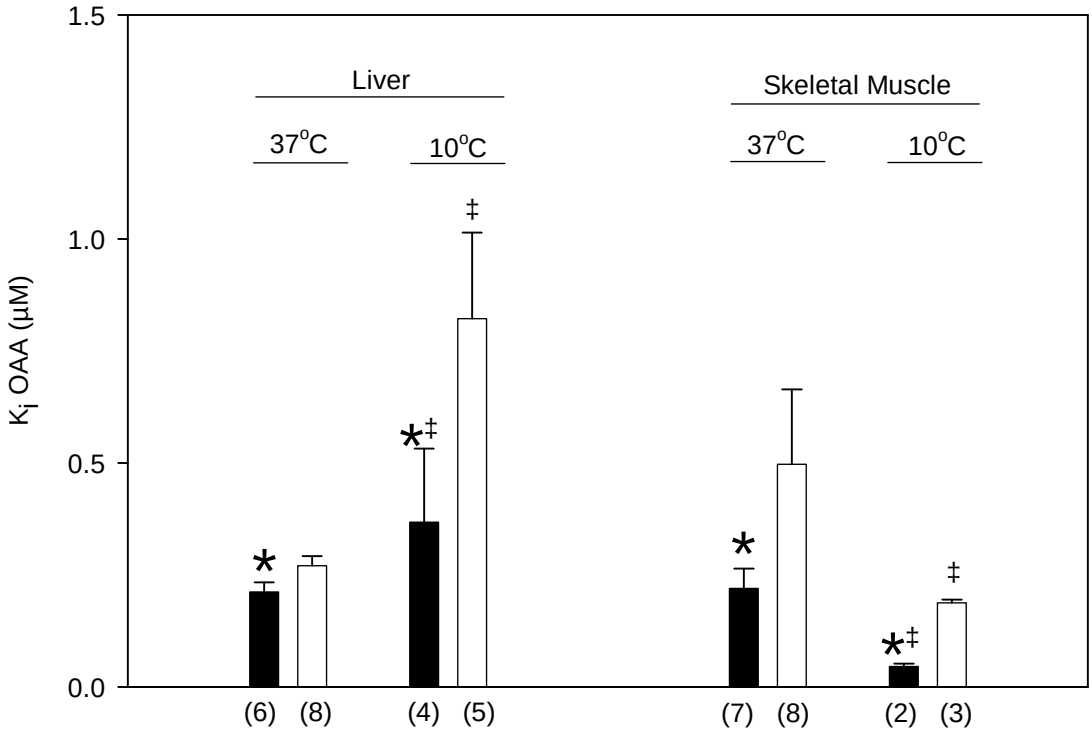
C
Liver 10°C
SDH Activity
(mU mg-1 protein)
(7)
(6)
State P = 0.38
State x Succinate P = 0.87
0
5
10
15
20
0
2
4
6
8
10
[Succinate] (mM)


D
Skeletal Muscle 10°C
(8)
(6)
State P = 0.11
State x Succinate P = 0.98
0
10
20
30
40
50
0
2
4
6
8
10
[Succinate] (mM)


1.0
0.8
0.6
0.4
0.2
0.0
Correlation Coefficient (r^2)
0
2
4
6
8
10
[Succinate] (mM)
*
**
*
*
**
*
*
*
**


A
Liver
Skeletal Muscle
37°C
10°C
37°C
10°C
AppK$_M$ Succinate (mM)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
**
**
(8) (7)
(2) (4)
(6) (6)
(4) (3)


B
Liver
Skeletal Muscle
37°C
10°C
37°C
10°C
AppK$_M$ Succinate (mM)
0.4
0.3
0.2
0.1
0.0
*
*
‡
‡
(11) (8)
(6) (7)
(7) (8)
(8) (6)


1.5
1.0
0.5
0.0
K_i OAA (μM)
Liver
Skeletal Muscle
37°C
10°C
37°C
10°C
*
*‡
*
*‡
‡
‡
(6) (8) (4) (5) (7) (8) (2) (3)