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ARTICLE *in* JOURNAL OF EXPERIMENTAL ZOOLOGY PART A ECOLOGICAL GENETICS AND PHYSIOLOGY · NOVEMBER 2015

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RESEARCH ARTICLE

Mitochondrial Phenotype of Marsupial Torpor: Fuel Metabolic Switch in the Chilean Mouse–Opossum *Thylamys Elegans*

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ABSTRACT

Torpor is a phenotype characterized by a controlled decline of metabolic rate and body temperature. During arousal from torpor, organs undergo rapid metabolic reactivation and rewarming to near normal levels. As torpor progress, animals show a preference for fatty acids over glucose as primary source of energy. Here, we analyzed for first time the changes in the maximal activity of key enzymes related to fatty acid (Carnitine palmitoyltransferase and β-Hydroxyacyl CoA dehydrogenase) and carbohydrate (Pyruvate kinase, Phosphofructokinase and Lactate dehydrogenase) catabolism, as well as mitochondrial oxidative capacity (Citrate synthase), in six organs of torpid, arousing and euthermic Chilean mouse-opossums (*Thylamys elegans*). Our results showed that activity of enzymes related to fatty acid and carbohydrate catabolism were different among torpor phases and the pattern of variation differs among tissues. In terms of lipid utilization, maximal enzymatic activities differ in tissues with high oxidative capacity such as heart, kidney, and liver. In terms of carbohydrate use, lower enzymatic activities were observed during torpor in brain and liver. Interestingly, citrate synthase activity did not differ thought torpor-arousal cycle in any tissues analyzed, suggesting no modulation of mitochondrial content in *T. elegans*. Overall results provide an indication that modulation of enzymes associated with carbohydrate and fatty-acid pathways is mainly oriented to limit energy expensive processes and sustain energy metabolism during transition from torpor to euthermy. Future studies are required to elucidate if physiological events observed for *T. elegans* are unique from other marsupials, or represents a general response in marsupials. J. Exp. Zool. 9999A:XX–XX, 2015. © 2015 Wiley Periodicals, Inc.

How to cite this article: Cortes PA, Bacigalupe LD, Mondaca F, Desrosiers V, Blier PU. 2015. Mitochondrial phenotype of marsupial torpor: Fuel metabolic switch in the Chilean mouse–opossum *Thylamys elegans*. J. Exp. Zool. 9999A:XX–XX.

J. Exp. Zool. 9999B:XX–XX, 2015

The maintenance of thermal homeostasis can be a major challenge for endotherms when environmental conditions are energetically restrictive (i.e., periods of low environmental temperature and/or food scarcity). Physiological dormancy or torpor is a highly successful adaptation employed by various mammalian species to cope with these unfavourable conditions (Geiser and Kenagy, 1998; Riek and Geiser, 2014). Torpor is a phenotype characterized by an almost complete suppression of expensive process with the aim of reducing energy expenditure (Carey et al., 2003; Melvin and Andrews, 2009; Wang and Lee, 2011). In therian mammals (i.e. marsupials and placental mammals), each torpor bout can last from a minimum of a single day (daily torpor) or up to several weeks (hibernation) (Geiser and Ruf, '95; Ruf and Geiser, 2014). Along winter, therian mammals experience multiple torpor bouts, each of these including three physiological states: deep torpor, arousal, and post-torpor normothermy (euthermy). Although during torpor animals experience a controlled reduction of metabolism, oxygen consumption and heart rate (Carey et al., 2003; Geiser, 2004), several aspects of the cardiovascular system (Milsom et al., '99), respiratory ventilation (McArthur and Milsom, '91), metabolic waste regulation (Anderson et al., '90), hepatic fuel utilization (Serkova et al., 2007) and the central nervous system (Drew et al., 2007) must continue during torpor.

During arousal from torpor, animals experience a drastic increase in body temperature, oxygen consumption, and blood perfusion (Fons et al., '97; Milsom et al., '99; Hampton et al., 2010). The rapid increase in temperature and revascularization experienced by arousing animals would provoke extensive pathophysiological conditions (e.g., ischemia-reperfusion injuries) leading to tissue necrosis in absence of a precise coordination of the rewarming process and oxygen supply (Drew et al., 2001; Lindell et al., 2005; Kurtz et al., 2006). The first line of metabolic increase involves heart and lungs, as the cardiovascular-respiratory system is in charge of re-activating blood flow through the remaining tissues and organs, particularly the liver (Hirshfeld and O'Farrell, '76; Hampton et al., 2010). In placental mammals, brown adipose tissue is also included in the first line of metabolic increase during arousal from torpor, since this organ is responsible for heat production during rewarming process. In non-placental mammals, however, thermogenesis is mainly the consequence of rapid and isometric muscular contractions. This metabolic activation induces an increase in kidneys activity that plays a key role in elimination of metabolic waste produced and accumulated during torpor (Lesser et al., '70; Geiser and Kenagy, '88). Hence, not all the tissues and organs are re-activated at the same rate during rewarming process.

An important distinction between torpid and euthermic animals is fuel utilization in metabolically active organs. As torpor progress, animals show a preference for fatty acids over glucose as primary source of energy (Buck and Barnes, 2000; Andrews, 2004; Roberts, 2011). Biochemical adjustments supporting this fuel metabolic switch includes modulating the activity of transporter and oxidative enzymes acting along fatty-acid pathways. Comparisons of active and hibernating mammals suggest a high fatty acid oxidation capacity during torpor (Geer et al., '77; Yacoe, '83a; Wickler et al., '87). For example, β-Hydroxyacyl CoA dehydrogenase (HOAD), a mitochondrial fatty acid oxidation enzyme, showed lower activities in active than hibernating big brown bat (*Eptesicus fuscus*) (Yacoe, '83a). On the other hand, high protein concentration (and putatively activity) of carnitine palmitoyltransferase (CPT) has been reported in torpid little brown bats (*Myotis lucifagus*) (Eddy et al., 2006). Interestingly, the capacity to transport fatty acids into mitochondria through CPT is considered as a rate-limiting step in β-oxidation (Kerner and Hoppel, 2000; McGuire et al., 2013). Hence, CPT modulation in mitochondria can have considerable effect on rates of fatty acid oxidation during torpor.

The inhibition of carbohydrate utilization pathway represents the other side of the coin in fuel metabolic switch experienced by torpid animals (Nestler, '91; Storey and Kelly, '95; Roberts, 2011; Storey, 2012). Regulation of glycolytic pathway is partly insured by phosphofructokinase (PFK) and pyruvate kinase (PK), both of which have lower activities during torpor (Yacoe, '83b; Brooks and Storey, '92; Storey, 2012). The regulation of lactate dehydrogenase (LDH) is critical to restore the nicotinamide adenine dinucleotide needed to carry out the anaerobic part of glycolysis as well as to allow the channeling of pyruvate to the proper pathway (aerobic or anaerobic) (Holbrook et al., '75; Tavoni et al., 2013). Furthermore, in the presence of large excess of lactate, LDH could promote gluconeogenesis in liver, which depends on ATP. Unfortunately, the modulation of LDH associated to mammalian torpor has been poorly studied. The scarce evidence available suggests a low modulation of LDH activity through

Grant sponsor: Programa de Mejoramiento de la Calidad y la Equidad de la Educación Superior; grant number: MECESUP AUS 1203; grant sponsor: JEB Travelling Fellowship; grant sponsor: Comisión Nacional de Investigación Científica y Tecnológica; grant number: FONDECYT 3150215; grant sponsor: Beca Doctoral de la Comisión Nacional de Investigación Científica y Tecnológica; grant number: CONICYT; grant sponsor: Fondo Nacional de Desarrollo Científico y Tecnológico; grant number: FONDECYT 1120461; grant sponsor: NSERC Discovery Grant; grant number: RGPIN 155926-2011.

Conflicts of interest: None.

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Received 21 July 2015; Revised 16 October 2015; Accepted 19 October 2015

DOI: 10.1002/jez.1994

Published online XX Month Year in Wiley Online Library (wileyonlinelibrary.com).

torpor-arousal cycle in mammals (Heldmaier et al., '99). Besides, studies performed on oxidative capacity of hibernating mammals have documented seasonal changes in Citrate synthase (CS) activity (Yacoe, '83a; Wickler et al., '91; Page et al., 2009). CS represents first enzyme of the tricarboxylic acid cycle, which is a proxy of mitochondrial oxidative capacity and mitochondrial content (Wiegand and Remington, '86).

Previous studies revealed that fatty-acid derived ketone bodies could also be used as fuel in the brain and heart, especially during torpor and rewarming (Rauch and Behrisch, '81; Krilowicz, '85; Andrews et al., 2009). Even more, it has been suggested that lipid oxidation could be supplemented to some extent by gluconeogenesis in some tissues (e.g., brain and liver) (Yacoe, '83b; Nestler et al., 2000). Hence, how enzymes activities are modulated appears to be more tissue-dependent than a systemic generalized pattern. Although mechanisms underlying fuel-selection switch have received considerable attention by researchers, all studies has been restricted to placental mammals. However, similar research performed on marsupials are urgently needed, especially considering the physiological differences at thermogenetic and metabolic level that exist between both mammal lineages (Renfree, '81; Geiser and Ruff, 1995; Nicol et al., '97; Cortes et al., 2014). Furthermore, focusing on a narrow taxonomic group could prevent any signal to identify an ancestral metabolic adaptation from evolutionary innovation concerning fuel metabolic switch underlying mammalians torpor phenotypes.

This study aimed at filling that gap by investigating whether the fuel metabolic switch that have been observed through torpor-arousal cycle in placental mammals is also revealed in a torpid marsupial. To do so, the maximal activity of key enzymes related to fatty acid (HOAD and CPT) and carbohydrate (LDH, PFK, and PK) catabolism, as well as mitochondrial oxidative capacity (CS), were compared in six organs of torpid, arousing and euthermic Chilean mouse-opossums (*Thylamys elegans*). As torpor progress, animals show a preference for fatty acids over glucose as primary source of energy (Buck and Barnes, 2000; Andrews, 2004; Roberts, 2011). Previous studies have shown that fatty acid derived ketone bodies could also be used as fuel in strict aerobic tissue such as brain and heart (Andrews et al., 2009). Hence, it can be predicted that HOAD activity will be increased in metabolically organs, particularly in brain and heart, during torpor and arousal. The capacity to transport fatty acids into mitochondria is considered a rate-limiting step of lipid oxidation. For this reason, we expected a concerted response in the activities of CPT and HOAD through torpor-arousal cycle in *T. elegans*. Also, anaerobic metabolism (indicated by the activity of LDH) should be depressed in the liver during torpor since the resulting lactate would need to be removed at the expenses of ATP. A similar prediction applies to ATP-dependent PFK activity (i.e., depressed during torpor) as carbohydrates must be preserved to fuel metabolism during arousal.

MATERIALS AND METHODS

Animals

Seventeen adult individuals of *Thylamys elegans* were captured in Central Chile (33° 29′S; 70° 56′W) during Austral Autumn with Sherman traps located in the ground and bated with bananas and tuna. After capture, animals were transported to the laboratory at Universidad Austral de Chile, Valdivia (39° 48′S, 73° 14′W), placed in plastic cages of 45×30×20 cm with 2 cm of bedding. Before torpor induction and tissue collection, all animals were maintained in a temperature-controlled room at 20 ± 1°C with a 12:12 photoperiod for 2 weeks. During this period, all animals were fed with water and a mix of mealworms and ground beef.

Torpor Induction and Tissue Collection

Previous to induce torpor, all active animals were weighted (± 0.1 g) and normothermic body temperature ($T_{b\ NOR}$) was measured using a copper-constantan thermocouple (Cole Parmer, Digi-Sense, USA) inserted into the cloaca. Subsequently, animals were transferred to a temperature-controlled chamber (Pitec Instrument, Chile) at 5 ± 1°C with a 12:12 photoperiod. The physiological state of animals was visually monitored each 1 hr to determine whether a given individual was torpid or active. When animals became torpid, we waited for approximately 12 hr to make a new body mass and body temperature measurement ($T_{b\ TOR}$). This period of time is in accordance with previous studies reporting daily torpor bout length (<20 hr) for *T. elegans* (Silva-Duran and Bozinovic, '99; Bozinovic et al., 2005; Bozinovic et al., 2007). Six torpid animals were sacrificed immediately after $T_{b\ TOR}$ measurements by a rapid decapitation. Non-sacrificed animals were transferred to a temperature-controlled room (20 ± 1°C) and copper-constantan thermocouple were inserted into the cloacae and attached to the tail with cloth tape. We make sure the manipulation procedure was as fast and non-invasive as possible. Seven arousing animals were sacrificed by rapid decapitation after they reached middle point between normothermic and torpid body temperature ($T_{b\ NOR} - T_{b\ TOR}/2$). Four post-torpor normothermic animals were sacrificed by rapid decapitation after reaching their initial normothermic body temperature ($T_{b\ EUT}$). Brain, heart, kidneys, liver, lung, and skeletal muscle were collected after euthanasia and immediately frozen in liquid nitrogen and maintained at −80°C. Samples were shipped to Université du Québec à Rimouski (Canada) on dry shipper at −80°C. Body mass and temperature are reported in Table 1.

Enzymatic Assays

All tissues were homogenized in 19 volumes of homogenization solution (10 mM HEPES, 50 mM potassium phosphate, and 0.5 mM EDTA; pH 7.4) using three 10 s bursts with a Tissue Tearor homogenizer (Biospec Products Inc., Bartlesville, OK). Maximal enzyme activities were measured at 24°C using an Envision Multilabel Plate Reader (Perkin Elmer, Waltham, MA)

Table 1. Descriptive statistics for body mass (M_b) and temperatures (T_b) of *T. elegans* measured during normothermy ($T_{b\ NOR}$), torpor ($T_{b\ TOR}$), and experimental stage ($T_{b\ STAGE}$).

	Torpor (6)		Arousal (7)		Euthermy (4)	
Trait	Mean	s.e.m	Mean	s.e.m	Mean	s.e.m
$M_{b\ NOR}$ (g)	27.87	1.75	25.37	2.26	27.35	2.61
$M_{b\ STAGE}$ (g)	26.5	1.59	25.08	1.02	23.88	0.63
$T_{b\ NOR}$ (°C)	32.60	0.48	33.2	0.37	35.05	1.09
$T_{b\ TOR}$ (°C)	7.00	0.26	8.42	0.74	7.30	0.74
$T_{b\ STAGE}$ (°C)	7.00	0.26	20.69	0.29	32.4	1.16

spectrophotometer according to assay conditions in Thibeault et al. ('97). Enzyme activities were measured at only one temperature because our objective was to compare maximal activities in different tissues and conditions (i.e., torpor, arousal, euthermy) to detect up or down modulation of activities. In these conditions, maximal activity is a proxy of both the content and catalytic capacity of the different enzymes. Furthermore, considering the limitation in tissue availability, limiting measurement at only one temperature (24°C) allowed us to make replications of measurements and to maximize the number of enzymes that we measured. All assays were run in triplicate and specific activities were expressed as μmol min^{-1} mg^{-1} protein (Bradford protein-dye binding assay; Bradford ('76)). All enzymes are expressed and analyzed as CS-specific activities (normalized per unite of CS activity in the tissue). This normalization with CS allowed estimation of the content of different enzymes relative to the mitochondrial content of the tissues as well as the relative response of each enzyme to the physiological status of the animal.

Conditions for enzyme assays were as follows:

(1) Carnitine palmitoyltransferase (CPT): 75 mM TRIS-HCL, 1.5 mM EDTA, 0.25 mM DTNB, 0.035 mM palmitoyl CoA and 2 mM L-Carnitine; pH 8.0. Absorbance was measured at 412 nm. Homogenate was omitted for the control.
(2) β-Hydroxyacyl CoA dehydrogenase (HOAD): 100 mM triethanolamine -HCL, 5 mM EDTA, 1 mM KCN, 0.15, 0.2 mM NADH, and acetoacetyl CoA (omitted for the control); pH = 8.0. Absorbance was measured at 340 nm.
(3) Phosphofructokinase (PFK): 75 mM triethanolamine-HCL, 200 mM KCL, 6 mM $MgCL_2$, 2 mM AMP, 2.5 mM ATP, 0.16 mM NADH, 5 mM F-6-P, 10 U/mL TPI, 2 U/mL G-3-P, 2 U/mL Aldolase; pH = 8.0. Absorbance was measured at 340 nm. Homogenate was omitted for the control.
(4) Pyruvate kinase (PK): 50 mM Imidazile-HCL, 100 mM KCL, 10 mM $MgCL_2$, 5 mM ADP, 5 mM PEP, 0.15 mM NADH, 0.6 U/mL LDH; pH = 7.4. Absorbance was measured at 340 nm. Homogenate was omitted for the control.
(5) Lactate dehydrogenase (LDH): 100 mM potassium phosphate, 0.16 mM NADH, and 0.4 mM pyruvate (omitted for the control); pH 7.0. Absorbance was measured at 340 nm. For this assay, an additional tissue dilution was necessary (brain 1:50, heart 1:100, kidney 1:100, liver 1:200, lung 1:50, and muscle 1:300).
(6) Citrate synthase (CS): 10 mM potassium phosphate, 20 mM HEPES, 0.1 mM DTNB, 0.3 mM acetyl-coA, and 0.5 mM oxaloacetate; pH 8.1. Absorbance was measured at 412 nm. Homogenate was omitted for the control.

Data Analysis

In this study, we are mainly interested in comparing the specific pattern of maximal enzyme activity through torpor-arousal cycle. We know a priori that specific activities will vary among tissues and, thus, an interaction between stage and tissue is expected. However, given our limited sample size and the lack of replicates in some combinations of levels (see below) we decided to investigate only the pattern of enzymatic activity within each tissue using a one-way ANOVA. Data from CPT and HOAD activity in lung and skeletal muscle was excluded because total information was lost during data exportation from Envision Multilabel Plate Reader, Perkin Elmer, Waltham, MA. The same issue explain the lack of information for brain CPT activity during arousal. The direction of changes in enzyme activity in torpid and arousal states compared to euthermy for each of the six tissues is represented in Table 2. The relationship between the activity of CPT and HOAD was examined using regression analysis. R platform 2.15 was used to perform statistical analysis (R Development Core Team 2009).

RESULTS

Fatty Acids Metabolism Enzymes

Heart ($F_{(2,10)} = 4.46$, $P < 0.01$) and liver ($F_{(2,8)} = 4.39$, $P = 0.05$) CPT activities showed significant differences through torpor-arousal cycle (Fig. 1). Liver CPT activity was higher in arousing compared to either torpid or euthermic animals, which did not differ. In heart,

Table 2. Summary table illustrating the significant and direction of changes in enzyme activity in torpid and arousal states compared to euthermy for each of the six tissues considered in the present study.

	Brain		Heart		Kidney		Liver		Lung		Muscle	
Enzyme	Torpor	Arousal	Torpor	Arousal	Torpor	Arousal	Torpor	Arousal	Torpor	Arousal	Torpor	Arousal
CPT	**	**	UP	UP	**	**	**	UP	N/A	N/A	N/A	N/A
HOAD	UP	UP	**	**	DOWN	DOWN	**	UP	N/A	N/A	N/A	N/A
PFK	DOWN	**	**	**	**	**	DOWN	DOWN	**	**	**	**
PK	**	**	**	**	**	**	**	**	**	**	**	**
LDH	**	**	**	**	**	**	DOWN	**	**	**	**	**
CS	**	**	**	**	**	**	**	**	**	**	**	**

UP is increased enzymatic activity compared to euthermia: DOWN is decreased enzymatic activity compared to euthermia; N/A is activities non-computed.
**Is no change among physiological states.

activities increased from torpor to arousal, whereas normothermic animals showed the lowest activities. CPT activities in brain ($F_{(1,7)} = 3.21$, $P = 0.12$) and kidney ($F_{(2,9)} = 1.55$, $P = 0.26$) did not differ between physiological states (Fig. 1). For HOAD, brain ($F_{(2,12)} = 8.31$, $P < 0.01$), kidney ($F_{(2,9)} = 4.39$, $P < 0.05$) and liver ($F_{(2,9)} = 8.31$, $P < 0.01$), but not heart ($F_{(2,12)} = 1.14$, $P = 0.35$), showed significantly different activities through torpor-arousal cycle (Fig. 2). Brain HOAD activity was higher in torpid compared to arousing or either euthermic animals. The opposite pattern was observed in kidneys; with torpid animals showing lower activities compared to other physiological states. In liver, arousing animals showed higher HOAD activities compared to torpid or euthermic animals. CPT and HOAD activities were significantly correlated in heart ($r = -0.59$, $P = 0.05$) and liver ($r = 0.56$, $P < 0.05$), but not in brain ($r = -0.29$, $P = 0.42$) and kidney ($r = -0.20$, $P = 0.48$). Heart CPT and HOAD activities were negatively correlated, whereas in liver the activities of these enzymes were positively correlated.

Carbohydrate Metabolism Enzymes

In brain ($F_{(2,101)} = 3.88$, $P = 0.05$) and liver ($F_{(2,11)} = 9.40$, $P < 0.01$) PFK activities showed significant differences through torpor-arousal cycle (Fig. 3). Brain PFK activity was lower during torpor compared to euthermy, whereas arousal activity did not differ with the remaining physiological states. In liver, PFK activity was higher during euthermia compared with either torpid or arousing animals. PFK activities in heart ($F_{(2,12)} = 1.96$, $P = 0.18$), kidney ($F_{(2,12)} = 1.01$, $P = 0.39$), lung ($F_{(2,12)} = 1.55$, $P = 0.25$) or skeletal muscle ($F_{(2,9)} = 1.01$, $P = 0.39$) did not show any differences through torpor-arousal cycle. Interestingly, PK was the only enzyme without any differences among stages in all organs evaluated (brain: $F_{(2,11)} = 2.49$, $P = 0.13$, heart: $F_{(2,12)} = 3.03$, $P = 0.09$, kidney: $F_{(2,13)} = 0.40$, $P = 0.68$, liver: $F_{(2,12)} = 1.88$, $P = 0.20$, lung: $F_{(2,12)} = 1.55$, $P = 0.25$, skeletal muscle: $F_{(2,12)} = 1.36$, $P = 0.30$). For LDH activity, liver ($F_{(2,13)} = 8.96$, $P < 0.01$) was the only organ showing a significant difference (brain: $F_{(2,13)} = 0.54$, $P = 0.60$, heart: $F_{(2,8)} = 0.56$, $P = 0.59$, kidney: $F_{(2,13)} = 0.70$, $P = 0.51$, lung: $F_{(2,11)} = 1.65$, $P = 0.24$, skeletal muscle: $F_{(2,8)} = 0.70$, $P = 0.75$) (Fig. 4). Torpid animals showed lower liver LDH activity compared to arousing or either euthermic animals.

TCA Cycle Enzyme

CS activity did not differ trough torpor-arousal cycle in any organ analysed (brain: $F_{(2,14)} = 0.24$, $P = 0.79$, heart: $F_{(2,12)} = 0.48$, $P = 0.63$, kidney: $F_{(2,14)} = 0.02$, $P = 0.98$, liver: $F_{(2,13)} = 0.49$, $P = 0.62$, lung: $F_{(2,13)} = 0.81$, $P = 0.47$, skeletal muscle: $F_{(2,14)} = 0.78$, $P = 0.48$) (Fig. 5).

DISCUSSION

In mammals, the transition to torpor is characterized by a fuel metabolic switch from a carbohydrate-based to lipid-based metabolism (Nestler, '91; Geer et al., '77; Buck and Barnes, 2000;

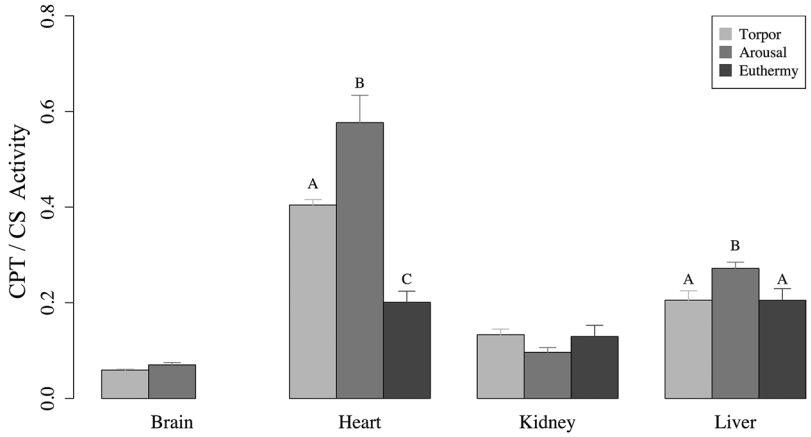

Figure 1. Maximal activity of carnitine palmitoyltransferase (CPT) in four metabolically active organs of torpid, arousing and euthermic Chilean mouse-opossums. CPT activity (μmol min^{-1} mg^{-1} protein) is expressed as CS-specific activities (normalized per unite of CS activity in the tissue). Values are expressed in means ± s.e.m. Means with different letter are significantly different (Tukey's HSD, $P < 0.05$).

Andrews 2004; Roberts 2011). In the present study, the maximal activity of key enzymes related to fatty acid (CPT and HOAD) and carbohydrate (PFK, PK and LDH) catabolism, as well as mitochondrial oxidative capacity (e.g., CS), were compared in six organs of torpid, arousing and euthermic Chilean mouse-opossums. Enzyme activities were different among torpor phases and the pattern of variation differs among tissues. In general, maximal activity of enzymes related to lipid utilization differs in tissues with high oxidative capacity such as, heart, kidney, and liver. In terms of carbohydrate use, lower enzymatic activities were observed during torpor in brain and liver. Interestingly, CS activity did not differ thought torpor-arousal cycle in any tissues analyzed, suggesting no modulation of mitochondrial content in *T. elegans*. Unfortunately, our interpretations are entirely limited to CS (initial steps of TCA cycle) and modulation of mitochondrial oxidative capacity could be provided by adjustment of others key enzymes or limiting steps, for example the electron transport system.

The liver is one of the most metabolically active organs in mammals; in adult animals, it is responsible for ~20% of metabolic rate (Martin and Fuhrman, '55). In fact, the active suppression of liver respiration can explain up to 70% of

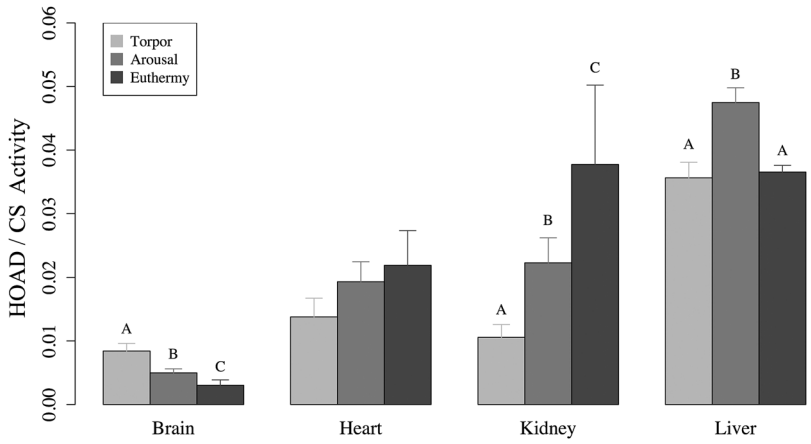

Figure 2. Maximal activity of β-Hydroxyacyl CoA dehydrogenase (HOAD) in four metabolically active organs of torpid, arousing and euthermic Chilean mouse-opossums. HOAD activity (μmol min^{-1} mg^{-1} protein) is expressed as CS-specific activities (normalized per unite of CS activity in the tissue). Values are expressed in means ± s.e.m. Means with different letter are significantly different (Tukey's HSD, $P < 0.05$).

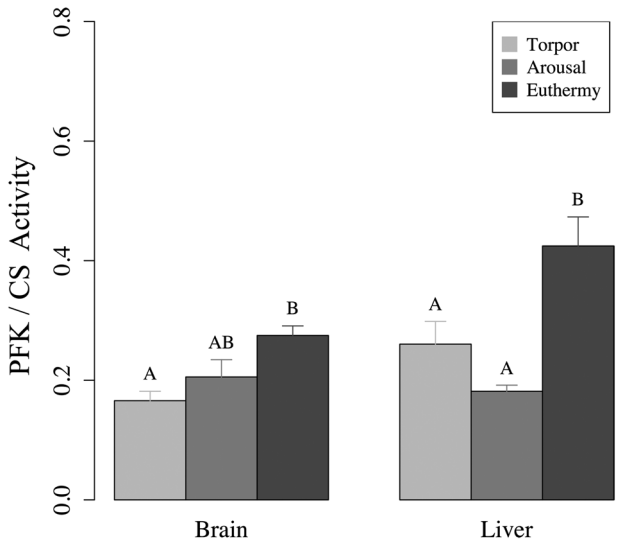

Figure 3. Maximal activity of Phosphofructokinase (PFK) in brain and liver of torpid, arousing and euthermic Chilean mouse-opossums. PFK activity (μmol min^{-1} mg^{-1} protein) is expressed as CS-specific activities (normalized per unite of CS activity in the tissue). Values are expressed in means ± s.e.m. Means with different letter are significantly different (Tukey's HSD, $P < 0.05$).

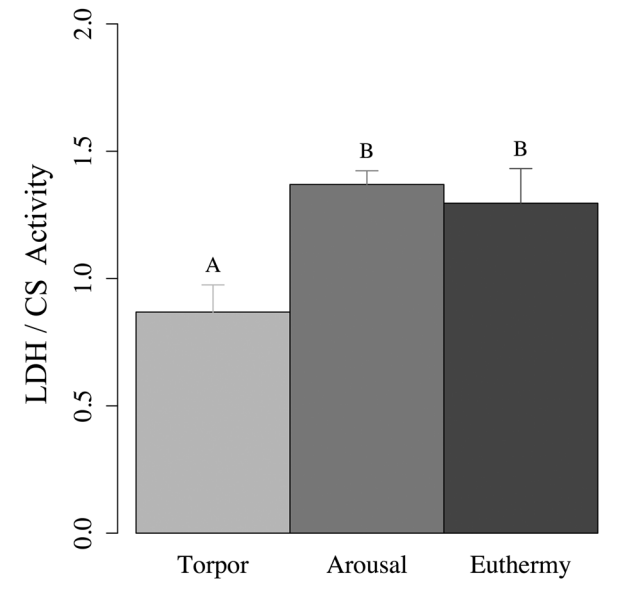

Figure 4. Maximal activity of Lactate dehydrogenase (LDH) in liver of torpid, arousing and euthermic Chilean mouse-opossums. LDH activity (μmol min^{-1} mg^{-1} protein) is expressed as CS-specific activities (normalized per unite of CS activity in the tissue). Values are expressed in means ± s.e.m. Means with different letter are significantly different (Tukey's HSD, $P < 0.05$).

metabolic rate reduction experienced by torpid animals (Armstrong and Staples, 2010; Brown et al., 2013). This tissue also plays a critical role in maintaining systemic lipid and glucose homeostasis during torpor. As torpor progresses and glycogen reserves are depleted, the liver oxidizes fat to provide both energy and substrate for gluconeogenesis (Staples and Hochachka, '98; Nestler et al., 2000). Our results shows that maximal activity of both PFK and LDH were reduced in torpid Chilean mouse-opossums, coinciding with previous studies demonstrating decreased aerobic and anaerobic glycolytic capacities during torpor in placental mammals (Yacoe, '83b; Brooks and Storey, '92; Storey, 2012). Interestingly, the reactions catalyzed by PFK and LDH, but not PK, are energy -requiring process (Berg et al., 2006). Whereas aerobic glycolysis carried out by PFK is an ATP-dependent process, reactions catalyzed by LDH under anaerobic conditions depend on NADH supply. Coordinated regulation of PFK and LDH might also be required to maintain a proper redox status of the NAD pool (NAD/NADH) during the different physiological conditions. Hence, our results could also indicate that glycolytic pathway is subject of selective regulation during torpor in order to limit energy expenditure. Regarding to fatty acid catabolism, liver CPT and HOAD maximal activity increased during arousal with no differences among torpid and euthermic animals. These results indicate that the potential for fatty acid oxidation in *T. elegans* liver is low during daily torpor, whereas an increased fatty acid oxidative capacity is required to properly fuel one of the most metabolically active organs during rewarming process.

The brain showed another pattern of response through torpor-arousal cycle. Maximal activity of PFK significantly decreased during both torpor and arousal compared to euthermy, whereas HOAD rose in brain of torpid Chilean mouse-opossums. This indicates that as torpor progresses, brain undergoes a fuel shift away from carbohydrates and toward lipids oxidation. The brain has high-energy requirements, and under normal circumstances, the predominant energy substrate is glucose (Sokolof, '73). This organ is particularly sensitive to hypoxia, which could lead to neuron degeneration and death in absence of appropriate hypoxia-response elements (Oosthuyse et al., 2001). However, it has been reported that production of ketone bodies from fatty acids may provide a neuro-protective mechanisms for preserve neural synaptic function in response to hypoxia (See Guzmán and Blázquez (2004) for a detailed review). Interestingly, recent findings suggest that fatty-acid derived ketone bodies can be used in replacement of glucose as fuel source during torpor (Rauch and Behrisch, '81; Krilowicz, '85; Andrews et al., 2009). Therefore, our results indicate that lipids are preferred over glucose as energy substrate by brain in torpid *T. elegans*. It is also possible that fatty acids might constitute a relevant substrate for ketone body formation by brain in torpid animals. If such is the case, future research is required to elucidate if fatty-acid derived ketone bodies contributes to sustain brain energy requirements

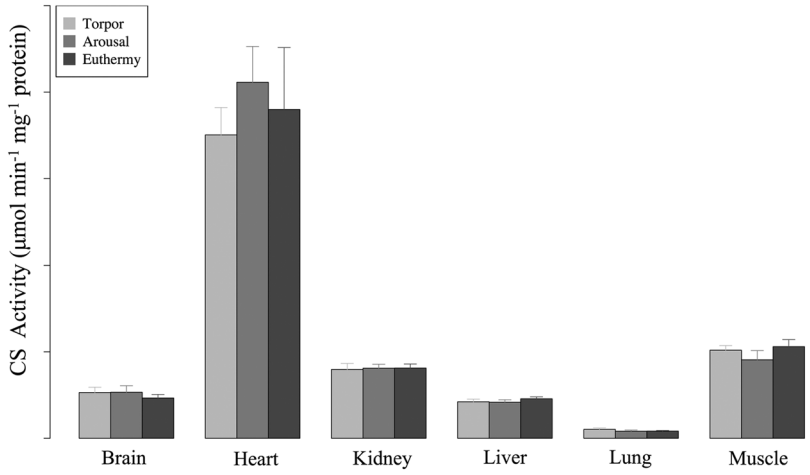

Figure 5. Maximal activity of Citrate synthase (CS) in six tissues of torpid, arousing and euthermic Chilean mouse-opossums. Values are expressed in means ± s.e.m.

and/or neuronal pro-survival response in torpid Chilean mouse opossums.

Typically, fatty acid oxidation rates are accelerated in heart of both torpid and arousing animals to ensure the appropriate blood pumping and circulation through torpor-arousal cycle (Andrews et al., '98; Yan et al., 2008). One potential site at which fatty acid oxidation may be regulated is at CPT, which is a rate-limiting step of β-oxidation (Kerner and Hoppel, 2000; McGuire et al., 2013). Previous studies have suggested that activation of CPT is also an essential element of cardio protection in response to ischemia-reperfusion episodes (Pauly et al., '91; Kantor et al., '99; He et al., 2012). Hence, the need to improve cardiac function and contractile performance through torpor-arousal cycle could lead to increased CPT and HOAD activities in heart of both torpid and arousing animals. Interestingly, however, while the capacity for mitochondrial fatty acid uptake was increased both during torpor and arousal, there was no corresponding variation in β-oxidative and glycolytic capacities in the heart of *T. elegans*. The observed increase in CPT activity may then suggest low room for metabolic adjustment through β-oxidation modulation leaving much of the control to substrate transport in heart.

During torpor-arousal cycle, small mammals undergo profound changes of renal function. Whereas urine production is drastically reduced during torpor, arousing animals experience a rapid recovery of kidney activity aimed to eliminate metabolic waste produced/stored during torpor and to restore water and electrolyte balance (Anderson et al., '90; Zancanaro et al., '99; Jani et al., 2011; Jani et al., 2013). Here, we observed that maximal activity of HOAD are increased in kidney of euthermic and aroused Chilean mouse-opossums compared to torpid animals. Our results are in accordance with previous studies documenting that mitochondrial oxidation of fatty acids represents a major source for renal energy production in conditions of high-energy demand and relatively little glycolytic capacity (Balaban and Mandel, '88; Bobulescu, 2010). This finding may indicate that regulation of enzymes related to B-oxidation is necessary to support energy requirements resulted from rapid restoring of renal functions during rewarming process.

Is the Oxidation Capacity Limited by Fatty-Acid Input to Mitochondria?

In this study, we considered CPT and HOAD activities as indicators of fatty-acid transport and β-oxidation capacity, respectively. Furthermore, it is known that the rate of β-oxidation could be limited by CPT activity (Kerner and Hoppel, 2000; McGuire et al., 2013). Contrary to our predictions, we did not find a clear and consistent relationship in the activity of both enzymes through torpor-arousal cycle. CPT and HOAD activities were correlated in heart and liver, but not in brain or kidney. Interestingly, the tissues showing a correlated activity are those with higher enzyme activity during arousal compared to torpor and euthermy. Whereas CPT and HOAD activities are positively correlated in liver, the opposite patter is observed for heart. The exact causes for these dissimilar patterns of co-variation are not known. In liver, it seems to exist a coordination between transport of fatty acids in mitochondria and β-oxidation capacity which might be independent of mitochondrial content (CS activity) but likely associated to modulation of fatty acid oxidation capacity. On the other hand, the inverse relation between CPT and HOAD in heart suggests a switch of the control of mitochondria oxidation of fatty acid (either transport or β-oxidation). Here, during rewarming an increase in fatty acid oxidation would be supported at the expense of a decrease in CPT activity (Barth et al., '92). Overall, our results highlight the role of

rate-limiting components of β-oxidation in tissues requiring high-energy input during arousal from torpor in marsupials (Kerner and Hoppel, 2000; McGuire et al., 2013).

CONCLUSIONS

Here, we presented the first evidence of flexibility in modulation of carbohydrate and fatty-acid catabolism pathways through adjustments of key enzymes activities in a marsupial species. How these enzymes activities are modulated in *T. elegans* support the classical evidence reported for placental mammals: as torpor progress, animals shows a preference for fatty acids over glucose as primary source of energy. In general, our results showed that during torpor, higher proportion of energy comes from fatty acids oxidation, whereas glucose is the main source of energy in euthermic animals (Buck and Barnes, 2000; Andrews, 2004; Roberts, 2011). Also, our results showed inhibition of carbohydrate oxidation in liver and brain during torpor in *T. elegans*. This is very important, as until today, these organs are assumed to be glucose-dependent. Finally, our findings adds support to previous works suggesting that fatty-acid derived ketone bodies could also be used as fuel in the brain and heart, especially during rewarming (Rauch and Behrisch, '81; Krilowicz, '85; Andrews et al., 2009).

Future Research on Biochemical Adjustments Underlying Marsupial Torpor

Marsupials and placental mammals represent two of the three major groups of modern mammals (Luo, 2007). Fossil record and molecular data suggest that marsupials and placental mammals shared the last common ancestor approximately 143–178 million years ago (Kumar and Hedges, '98, Woodburne et al., 2003). Although placental mammals and marsupials share many ancient mammalian characteristics, they have evolved distinctive physiological, anatomical and reproductive traits. The most distinct physiological difference in marsupials includes the absence of brown adipose tissue for non-shivering thermogenesis (Hayward and Lisson, '92). The data presented here demonstrated strong similarities among marsupials and placental mammals regarding to adjustments in metabolic pathways through torpor-arousal cycle. In this context, focusing on a narrow taxonomic group could prevent any signal to delineate if we observed ancestral characters or converging responses regarding metabolic adjustment among therian mammals. Further research is needed to determine whether mechanisms observed for *T. elegans* are different from other marsupials, or whether represents a general response in this lineage.

ACKNOWLEDGMENTS

We thank to Claudio Veloso, Pablo Sabat, Enrique Bazan, and Grisel Cavieres. P.A.C thanks to Programa de Mejoramiento de la Calidad y la Equidad de la Educación Superior [MECESUP AUS 1203], JEB Travelling Fellowship and Comisión Nacional de Investigación Científica y Tecnológica [FONDECYT 3150215]. P.A.C thanks to Servivio Agricola y Ganadero [SAG EXENTA 1291] for capture and maintenance permits. This work was supported by Beca Doctoral de la Comisión Nacional de Investigación Científica y Tecnológica [CONICYT to P.A.C], Fondo Nacional de Desarrollo Científico y Tecnológico [FONDECYT 1120461 to L.D.B] and NSERC Discovery Grant [RGPIN 155926-2011 to P.U.B].

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