
The Living Dead:
Mitochondria &
Metabolic Arrest
Kenstoreylab.com

HIBERNATION

13-LINED GROUND SQUIRREL

Ictidomys tridecemlineatus

HIBERNATION

Little Brown Bat
Myotis lucifugus

DAILY TORPOR

Gray mouse lemur
Microcebus murinus

FREEZING

Wood frog
Rana sylvatica

METABOLIC RATE DEPRESSION

Hibernation

Anoxia

Estivation

Freezing

Diapause

Consequences of hibernation

Body temperature ↓

Heart beat (1%) ↓

Respiration rate (3%) ↓

O_2 consumption ↓

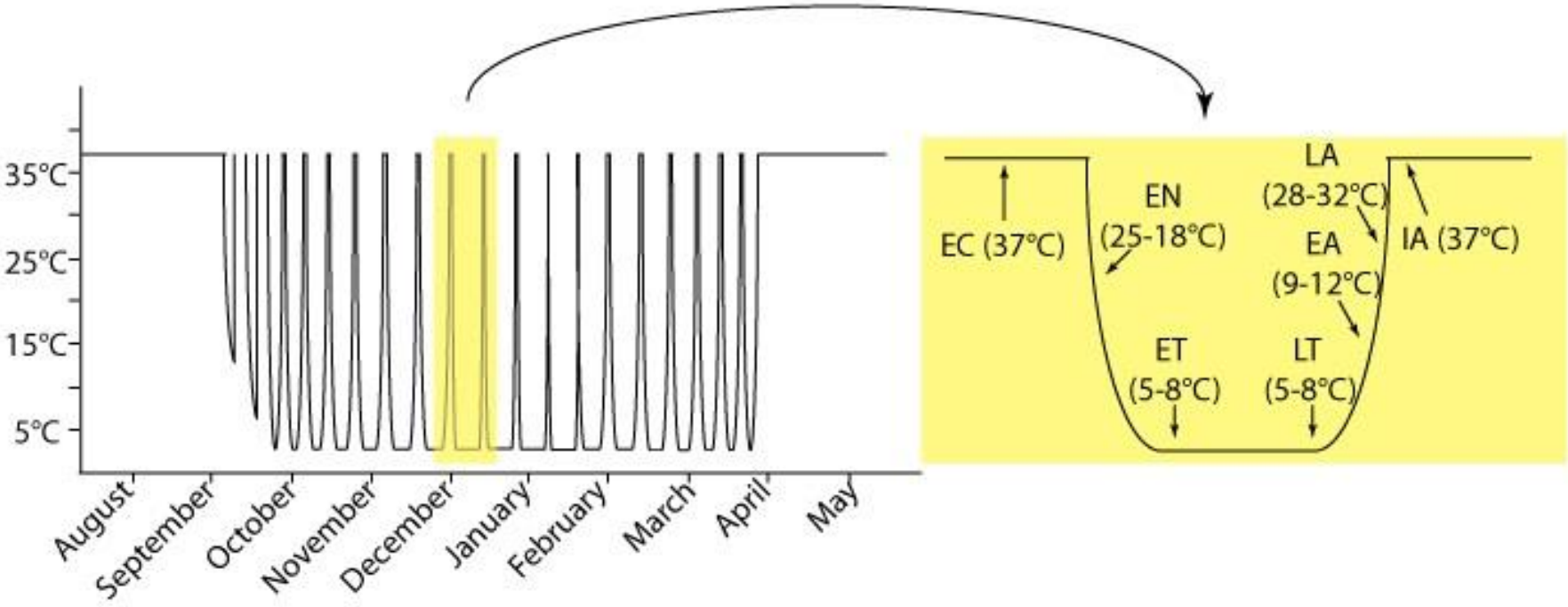
Cerebral blood flow (10%) ↓

Total energy savings: ~90%

Dramatic behavioral, physiological and biochemical changes.

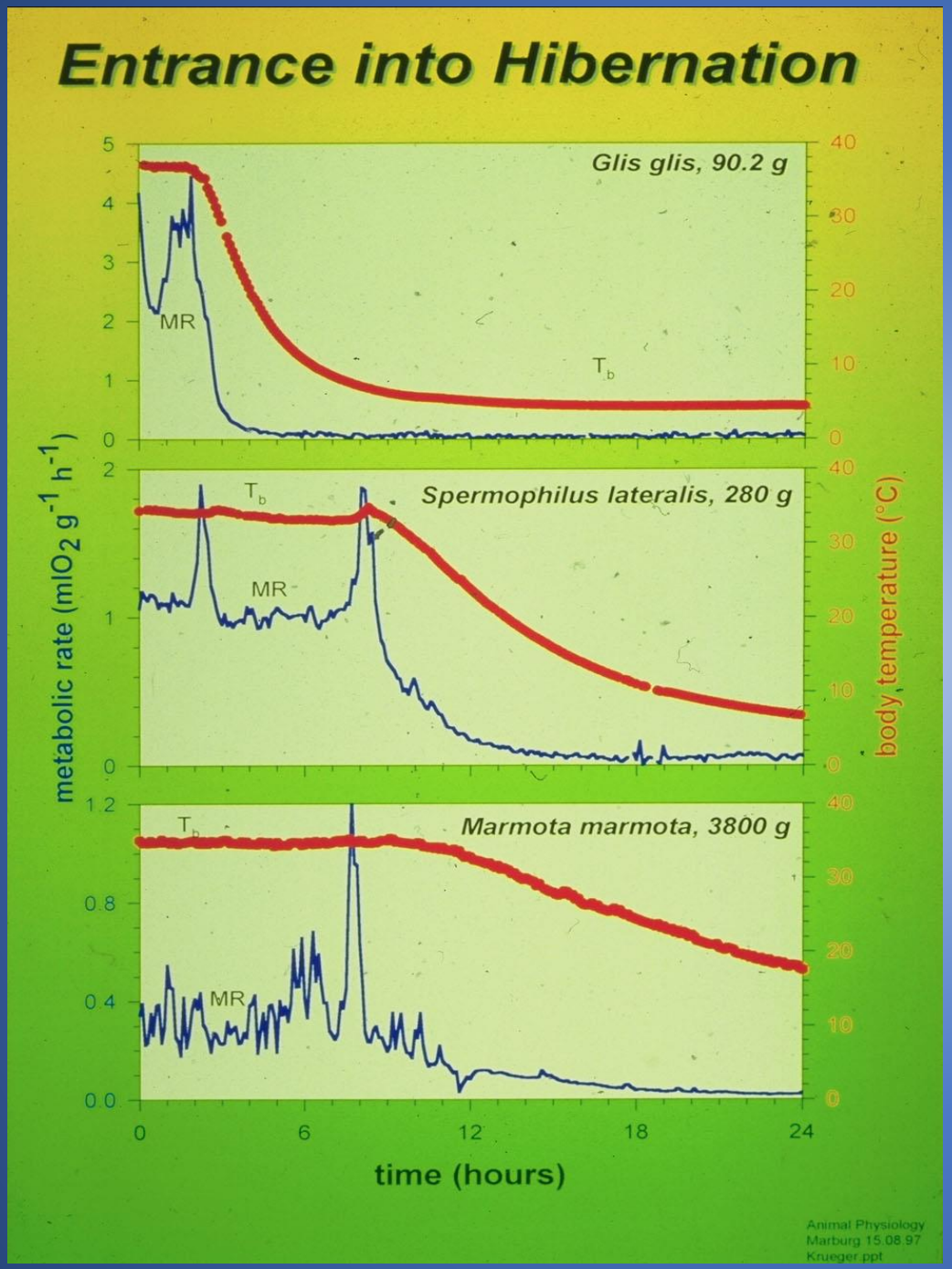
Photos: Nature Photography & J Am Med Assoc

TORPOR-AROUSAL

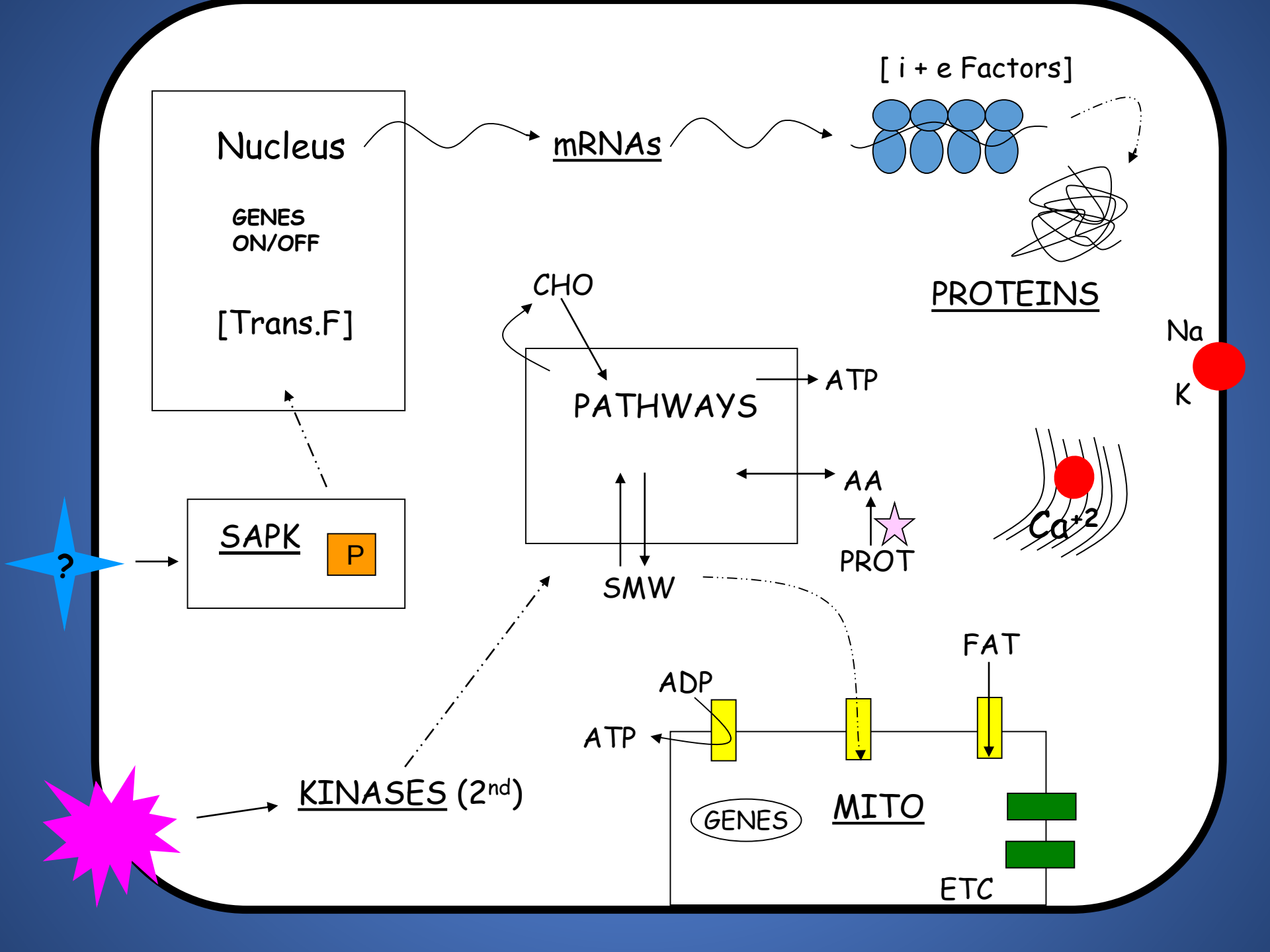


Animal studies by Dr. JM Hallenbeck and Dr. DC McMullen, NIH

Figure adapted from Nelson *et al.* 2009



- Metabolism inhibited causing Tb to fall
- Metabolic rate falls to <5% of normal
- Smaller animals cool down faster
- Q_{10} values up to 15
- Reversible in arousal
- Torpor bout duration 4 days to 2 weeks


[i + e Factors]
Nucleus
GENES
ON/OFF
[Trans.F]
mRNAs
PROTEINS
CHO
PATHWAYS
ATP
AA
PROT
SMW
Na
K
Ca+2
?
SAPK
P
KINASES (2nd)
ADP
ATP
FAT
GENES
MITO
ETC

PRINCIPLES OF HIBERNATION

1. Metabolic rate reduction
2. Control by protein kinases
   (SAPKs, 2nd messenger PKs)

[3. Most Genes OFF]

4. Selective gene activation

Differential expression of mitochondrial vs nuclear encoded subunits of cytochrome oxidase (complex IV) & ATP synthase (complex V)

The Journal of Experimental Biology 205, 1625–1631 (2002)
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JEB3879

Differential expression of mitochondria-encoded genes in a hibernating mammal

Dustin S. Hittel and Kenneth B. Storey*

Institute of Biochemistry and Department of Biology, Carleton University, 1125 Colonel By Drive, Ottawa, Ontario, Canada K1S 5B6

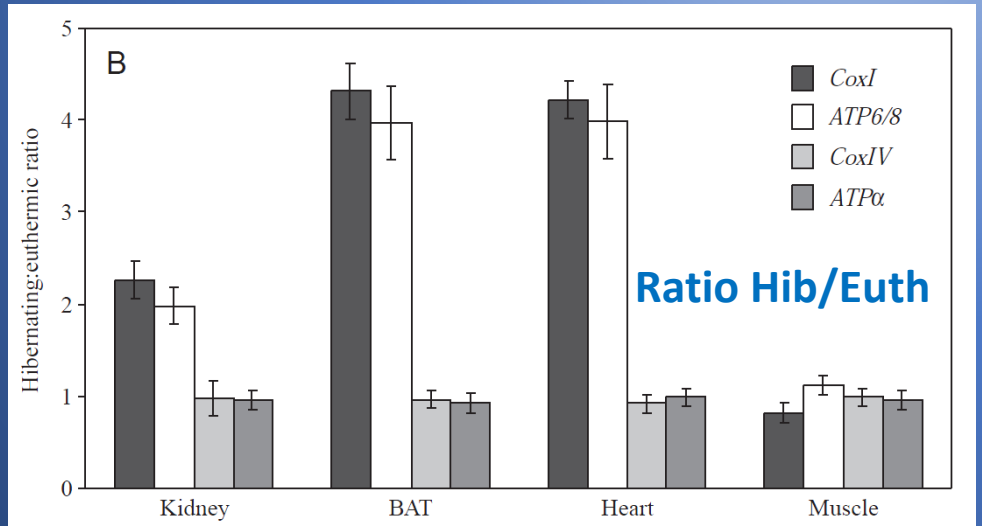
*Author for correspondence (e-mail: kenneth_storey@carleton.ca)

Accepted 13 March 2002

Summary

A cDNA library constructed from kidney of the thirteen-lined squirrel, *Spermophilus tridecemlineatus*, was differentially screened for genes that were upregulated during hibernation. A clone encoding cytochrome *c* oxidase subunit 1 was found and confirmed to have been upregulated by northern blotting. Differential expression of *Cox1* mRNA occurred in multiple organs during hibernation; in hibernating animals transcript levels were twofold higher in kidney and fourfold higher in heart and brown adipose tissue than in euthermic animals, but were unchanged in skeletal muscle. Transcript levels of mitochondrial-encoded ATP synthase 6/8 were similarly upregulated in these tissues whereas transcript levels of the nuclear encoded subunits *Cox4* and ATP synthase α did not change during hibernation. Immunoblot analysis revealed a 2.4-fold increase in Cox 1 protein and a slight decrease in Cox 4 protein in kidney of hibernating squirrels, compared with euthermic controls. Hibernating mammals may increase the expression of the mitochondrial genome in general, and *Cox1* specifically, to prevent or minimize the damage to the electron transport chain caused by the cold and ischemia experienced during a hibernation bout.

Key words: *Spermophilus tridecemlineatus*, hibernation, ischemia, kidney, cDNA library.



- Increased synthesis of mitochondria-encoded subunits in BAT, kidney & heart: *cox1 & ATP6/8*
- No change in synthesis of nuclear-encoded subunits in any tissue: *cox4 & ATPα*

Mitochondrial genes, proteins & enzyme activities increase during torpor in brown adipose of bats

JOURNAL OF EXPERIMENTAL ZOOLOGY 305A:620–630 (200

Differential Expression of Selected Mitochondrial Genes in Hibernating Little Brown Bats, *Myotis lucifugus*

SEAN F. EDDY*, PIER JR. MORIN, AND KENNETH B. STOREY
Institute of Biochemistry and Department of Chemistry Carleton University, Ottawa, Ont., Canada K1S 5B6

ABSTRACT High rates of non-shivering thermogenesis by brown adipose tissue accompanied by additional shivering thermogenesis in skeletal muscle provide the powerful reheating of body organs that allows hibernating mammals to return from their state of cold torpor back to euthermic function. Previous studies have suggested that changes to brown adipose mitochondria occur during hibernation and are partially responsible for its capacity for non-shivering thermogenesis. The current study shows that selected mitochondrial enzyme activities are elevated and selected genes and proteins are induced during torpor in brown adipose tissue of the little brown bat, *Myotis lucifugus*. Cytochrome oxidase activity in brown adipose tissue was more than 3-fold higher during torpor than in euthermic animals. Transcript levels of mitochondria-encoded genes, *coxII* and *nad4*, were also 3–4-fold higher during torpor, as evidenced by northern blotting. By contrast, transcripts of these genes were unchanged in skeletal muscle during torpor. Protein levels of carnitine palmitoyl transferase-1β, an enzyme embedded in the outer membrane of the mitochondria that is the rate-limiting step enzyme in β-oxidation, were also elevated by 2-fold during torpor in brown adipose but were unchanged in skeletal muscle. Cloning and sequencing of a 624 bp segment of *cpt-1β* revealed a number of amino acid substitutions in the bat protein as compared to CPT-1β from other mammals; these may be beneficial for enzyme function at low body temperatures during torpor. This study provides further evidence for a key role of mitochondria in hibernation. *J. Exp. Zool. 305A: 620–630, 2006.*

During torpor in brown adipose, compared with euthermia:

- **Cytochrome oxidase activity ↑ 3x**
- **Transcripts of *coxII* and *nad4* ↑ 3-4x -both mito-encoded genes**
- **Carnitine palmitoyl transferase-1b protein ↑ 2x**

Pyruvate Dehydrogenae Complex & Metabolic Rate Depression in Nature

Metabolic adjustments during daily torpor in the Djungarian hamster

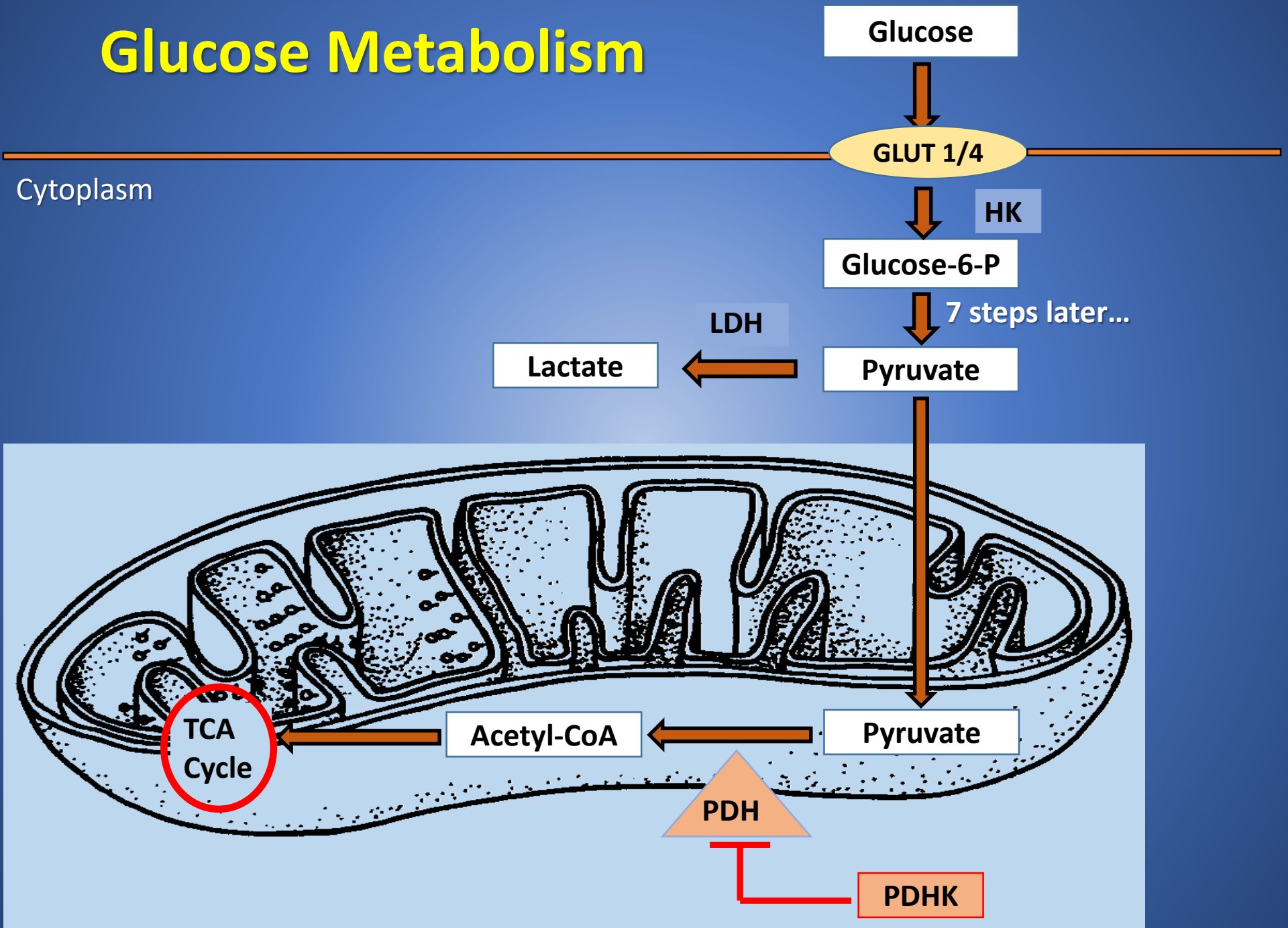
Heldmaier, Gerhard, Martin Klingenspor, Martin Werneyer, Brian J. Lampi, Stephen P. J. Brooks, and Kenneth B. Storey. Metabolic adjustments during daily torpor in the Djungarian hamster. *Am. J. Physiol.* 276 (*Endocrinol. Metab.* 39): E896–E906, 1999.—Djungarian hamsters (*Phodopus sungorus*) acclimated to a short photoperiod (8:16-h light-dark cycle) display spontaneous daily torpor with ad libitum food availability. The time course of body temperature (T_b), metabolic rate, respiratory quotient (RQ), and substrate and enzyme changes was measured during entrance into torpor and in deep torpor. RQ, blood glucose, and serum lipids are high during the first hours of torpor but then gradually decline, suggesting that glucose is the primary fuel during the first hours of torpor, with a gradual change to lipid utilization. No major changes in enzyme activities were observed during torpor except for inactivation of the pyruvate dehydrogenase (PDH) complex in liver, brown adipose tissue, and heart muscle. PDH inactivation closely correlates with the reduction of total metabolic rate, whereas in brain, kidney, diaphragm, and skeletal muscle, PDH activity was maintained at the initial level. These findings suggest inhibition of carbohydrate oxidation in heart, brown adipose tissue, and liver during entrance into daily torpor.

KEY ELEMENTS:

PDH major regulatory point

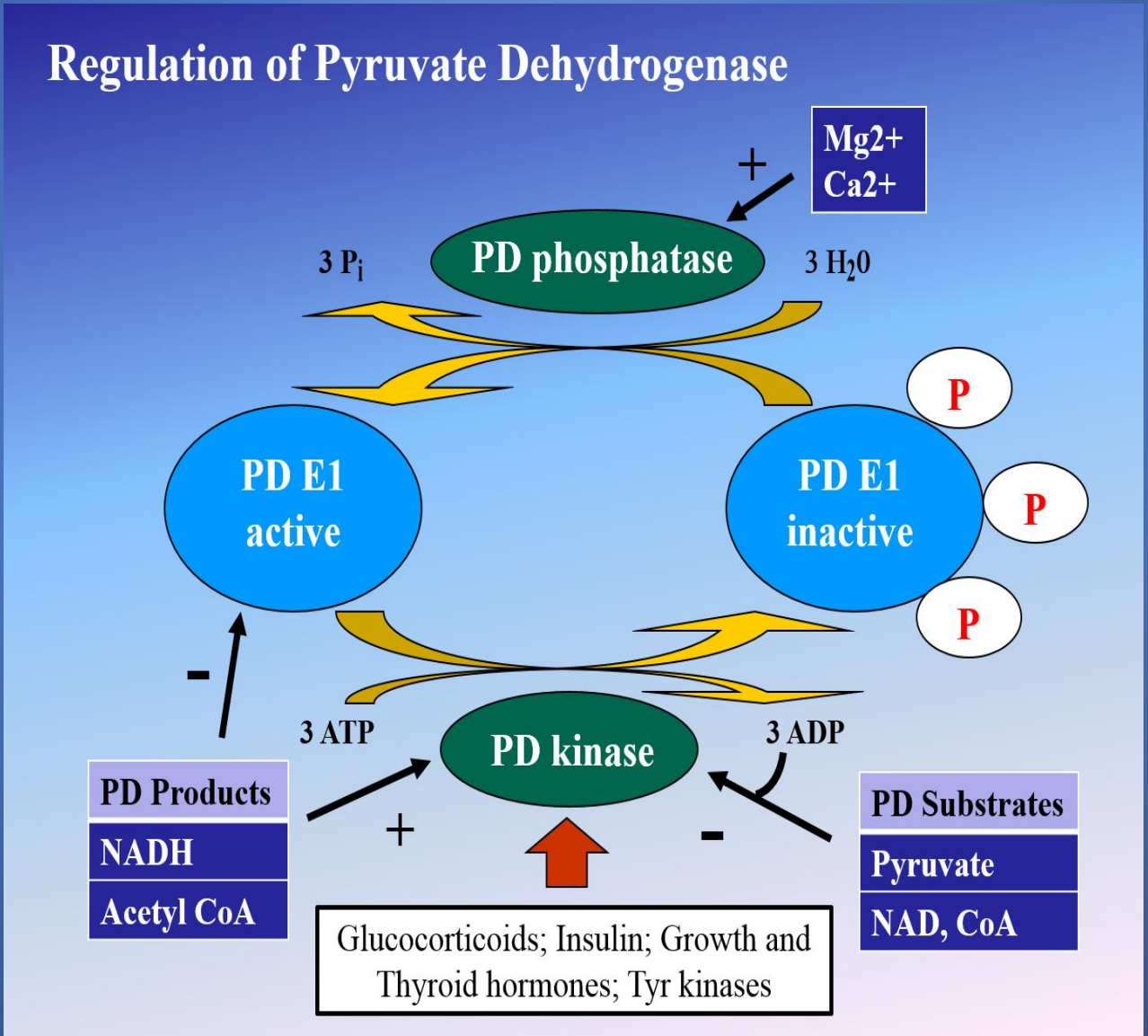
Inactivation correlates with MRD !

Glucose Metabolism

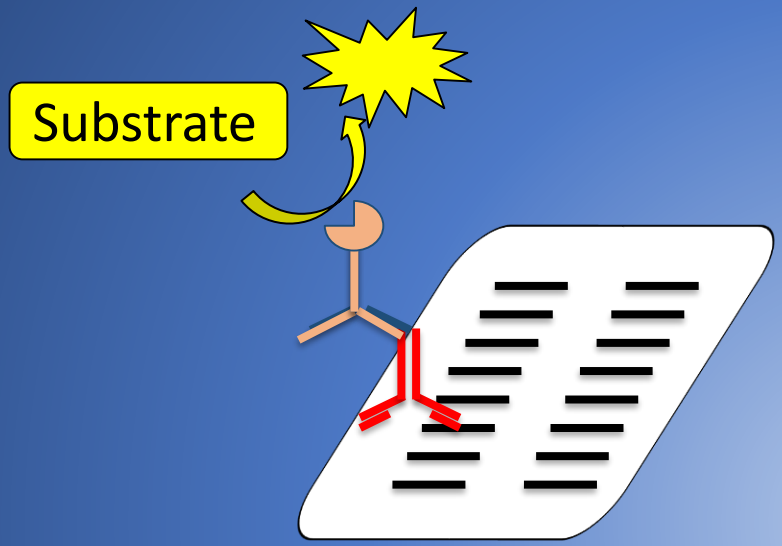


Phosphorylation of one or more Ser sites → INACTIVATES

pSer232, pSer293, pSer300



Methods



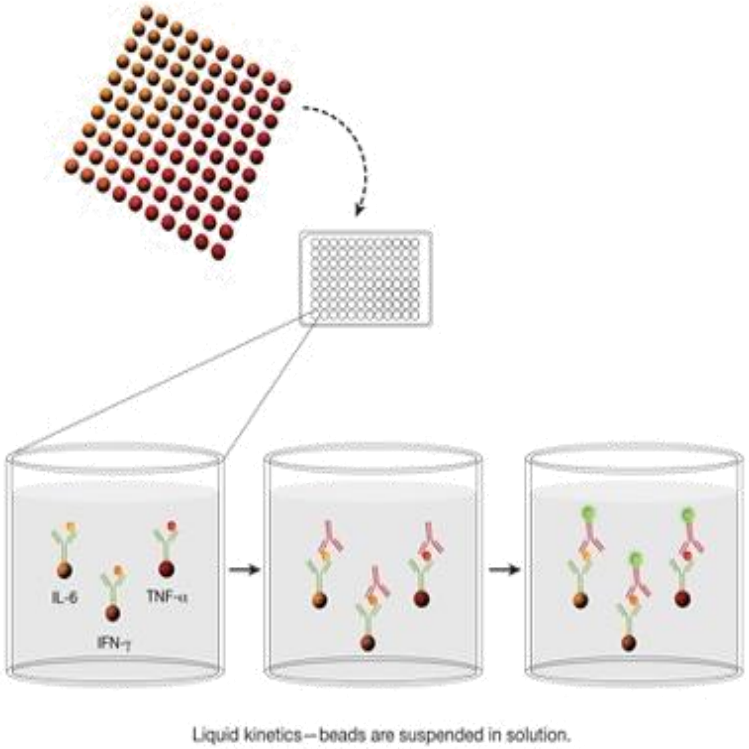
Western Blot

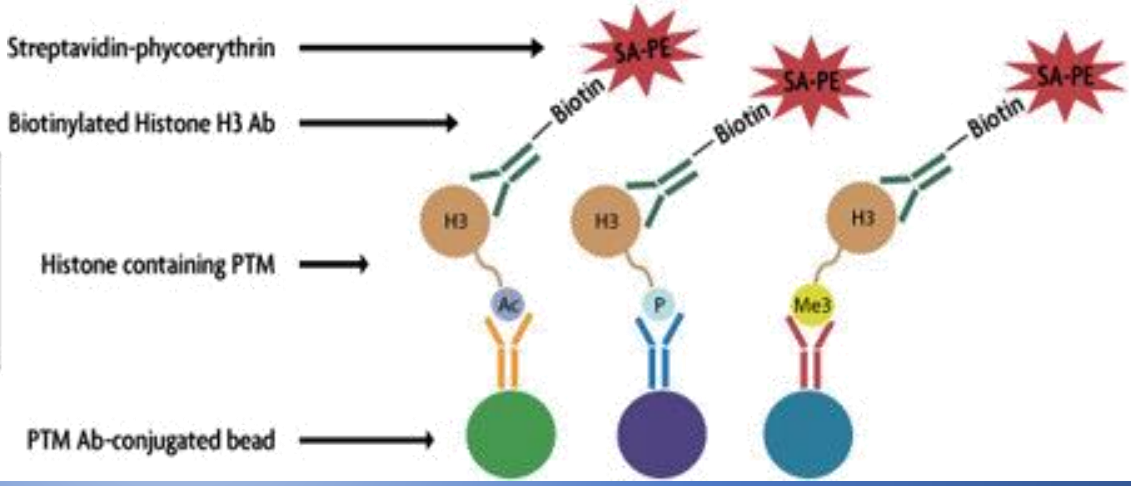
- **Proteins resolved on SDS- PAGE**
- **Proteins transferred to PVDF**
- **Antigens immobilized on membrane**
- **Antibody detects Antigens**
- **Visualization of DATA!**

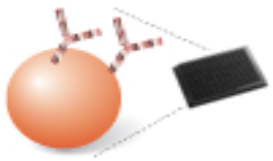
Luminex Multiplex

- High throughput
  - Western blots on **STEROIDS!**
- 96-well format
- Each well can measure up to 100 different targets
- Enzyme, Immuno, DNA & Receptor-ligand assays

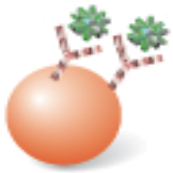
Luminex Technology



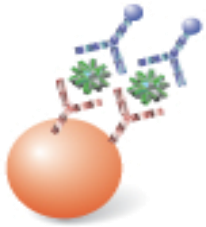




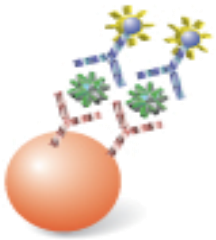
Step One:
- Dispense capture beads
- Wash plate 2 times



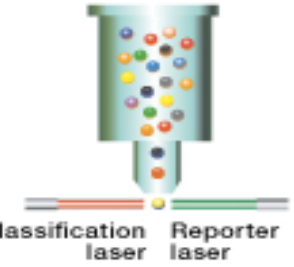
Step Two:
- Add samples
- Incubate
- Wash plate 3 times



Step Three:
- Add biotinylated detection antibody
- Incubate
- Wash plate 3 times

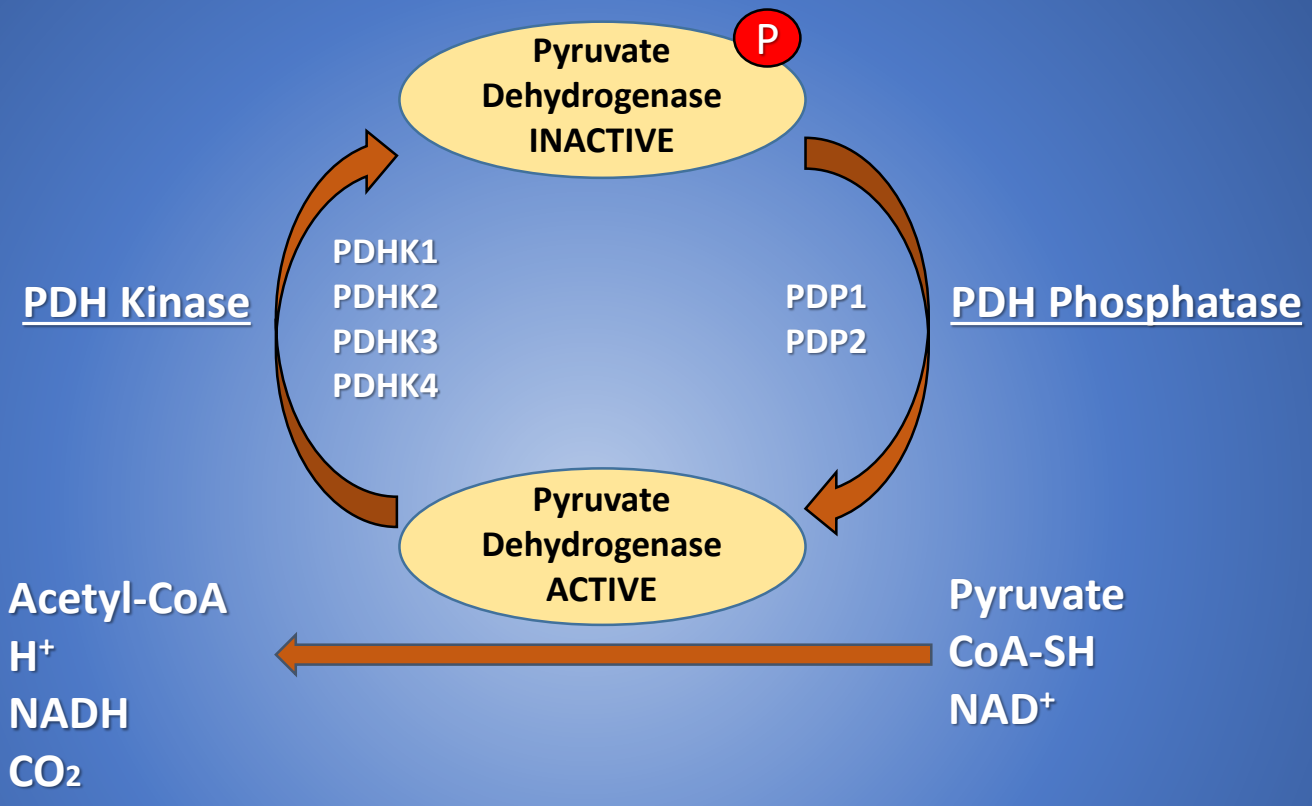


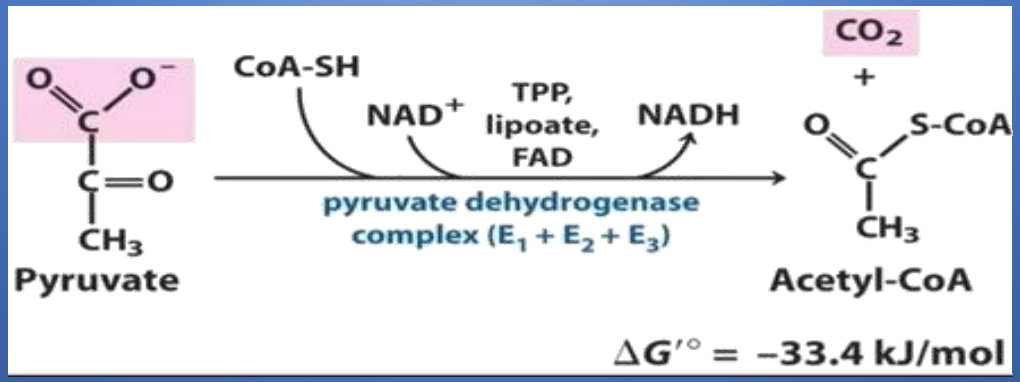
Step Four:
- Add streptavidin-PE reporter dye
- Incubate
- Wash plate 3 times



Step Five:
- Resuspend beads
- Perform fluorescent sorting
- Analyze data

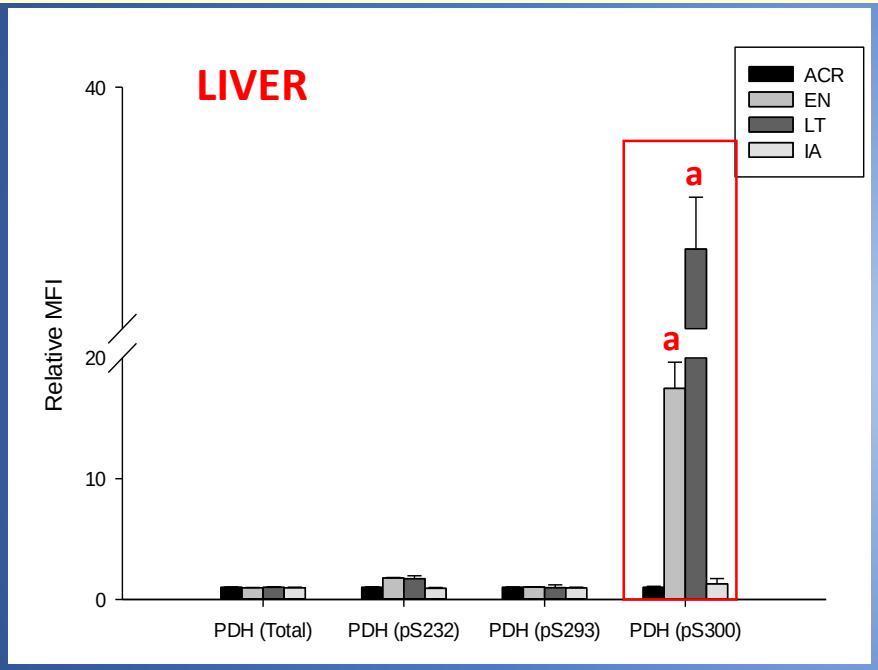
Pyruvate Dehydrogenase Regulation





PDH in 13LGS: Hibernation

Luminex compares:
active euthermic, entrance, late torpor, interbout arousal

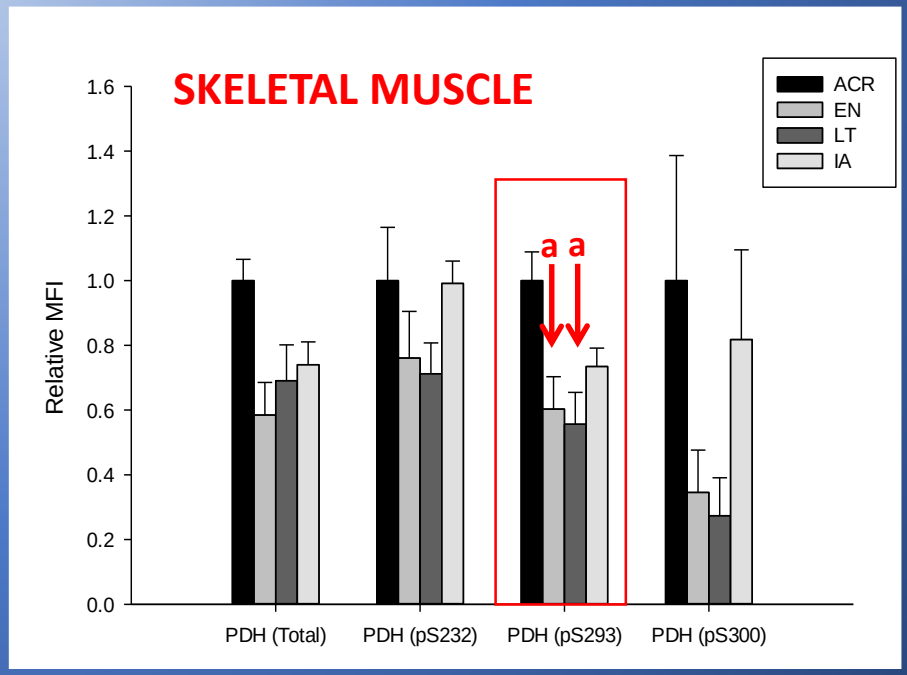


LIVER:

- During Torpor P-S300 ⇧
- No change in total PDH, P-S232, P-S293
- PDH activity is inhibited during torpor

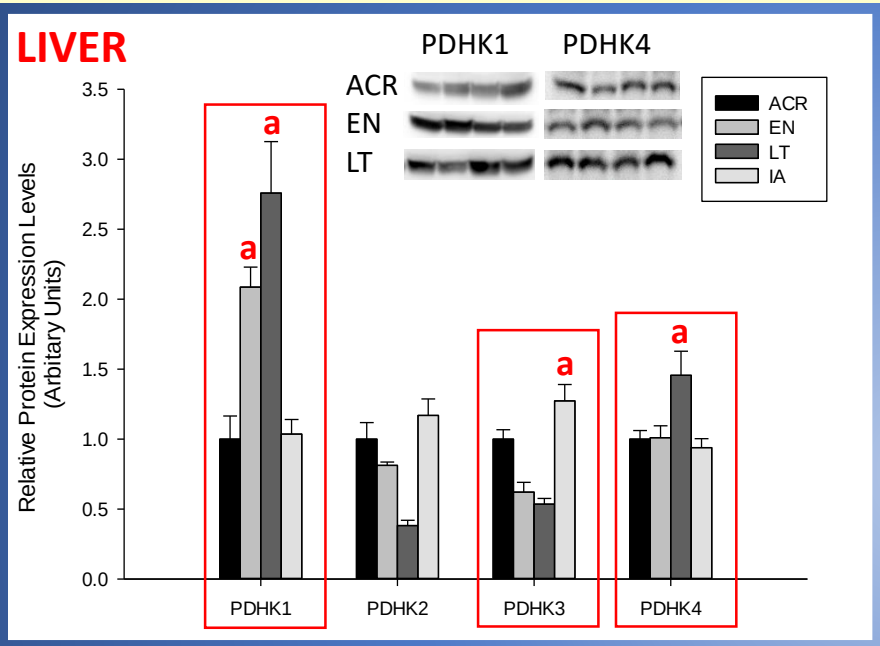
Skeletal Muscle:

- No change in total PDH, P-S232, and P-S300
- P-S293 ⇩
- Limited regulation of PDH during torpor



PDH-K in 13LGS: Hibernation

Immunoblotting: euthermic, entrance, torpor, arousal

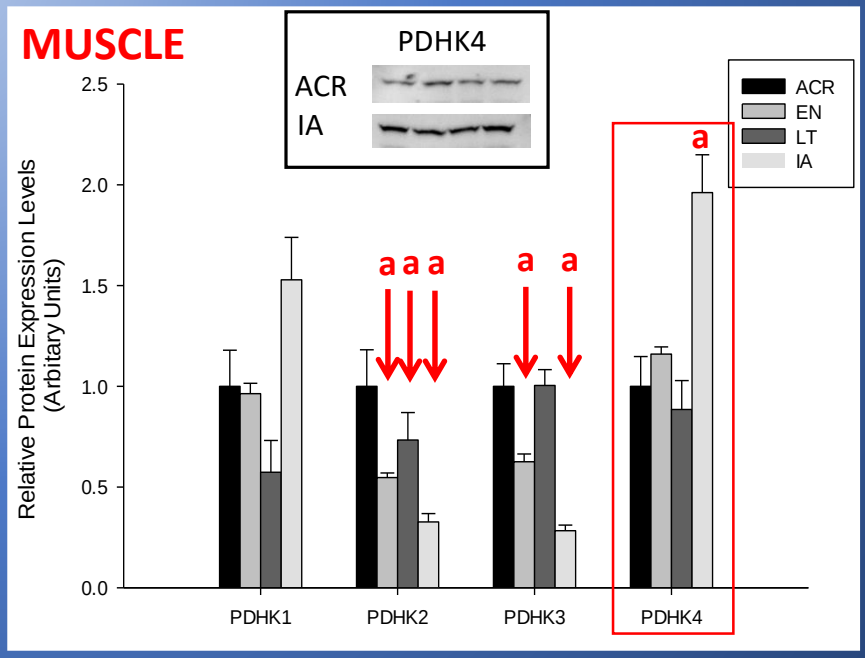


LIVER Hibernation:

- PDHK1, PDHK3, PDHK4 ↑
- Corresponds to p-PDH data
- PDH activity is inhibited during torpor

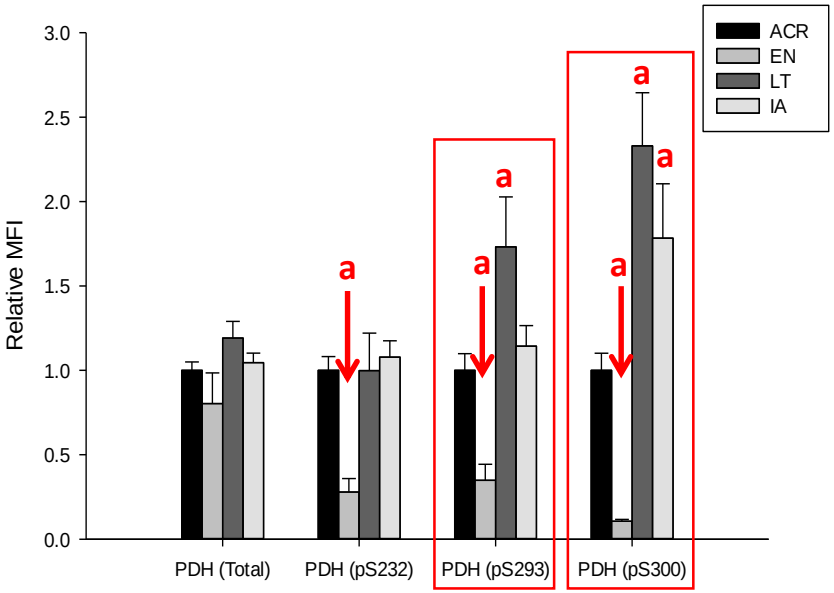
Skeletal Muscle:

- All PDHKs either reduced or do not change during torpor
- Limited regulation of PDH during torpor

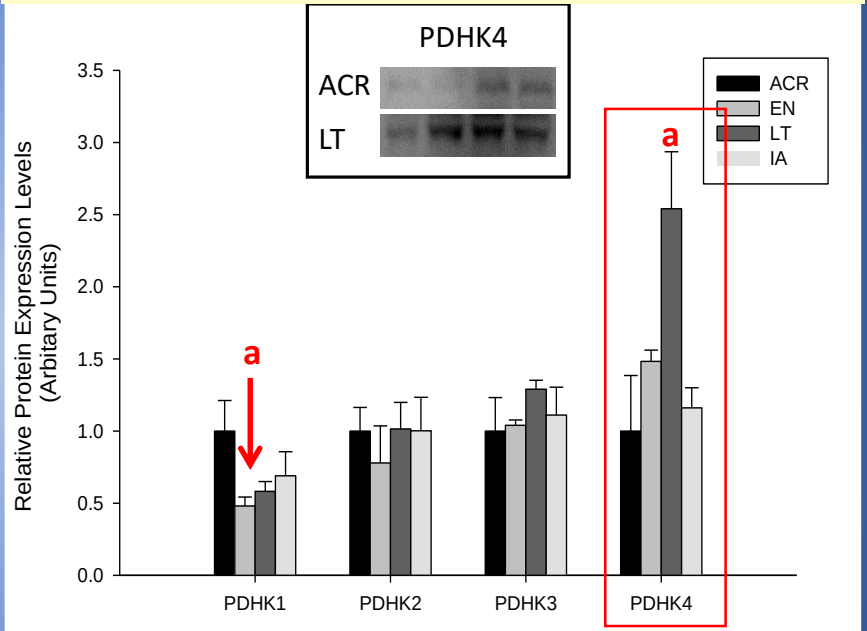


Heart PDH + PDHK in hibernation

Luminex analysis: euthermic, entrance, torpor, arousal



Immunoblotting: euthermic, entrance, torpor, arousal



- During torpor P-S293 and P-S300
- During entrance all phospho sites

- During torpor PDHK4
- Corresponds to P-S300 increase

PDH activity is inhibited during hibernation but may be active during entrance.

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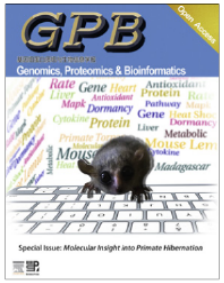


PREFACE

The Gray Mouse Lemur: A Model for Studies of Primate Metabolic Rate Depression

Kenneth B. Storey *,[a]

Institute of Biochemistry and Department of Biology, Carleton University, Ott

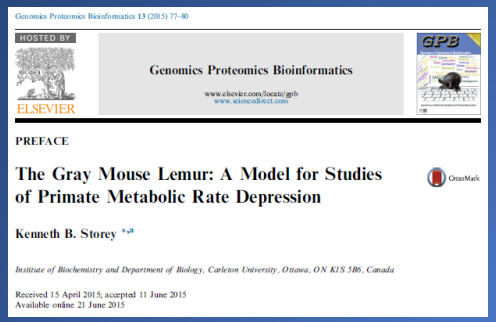
Received 15 April 2015; accepted 11 June 2015
Available online 21 June 2015

Gray mouse lemur, *Microcebus murinus*
- Native to Madagascar

PRIMATE HIBERNATION !!
Gray Mouse Lemur

Madagascar
- western dry
forests

Primate Torpor Series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PREFACE

The Gray Mouse Lemur: A Model for Studies of Primate Metabolic Rate Depression

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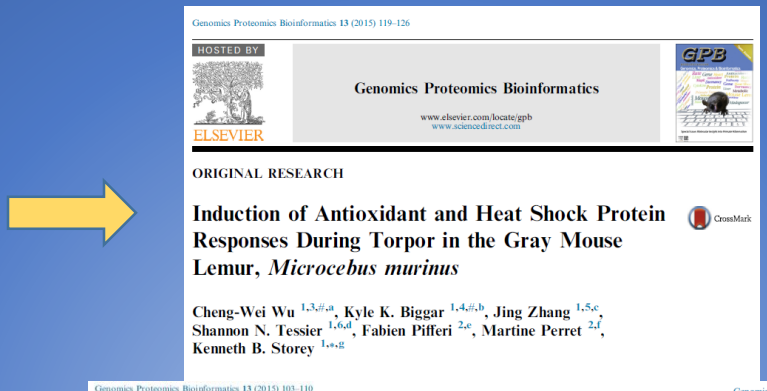
The stress response and signal transduction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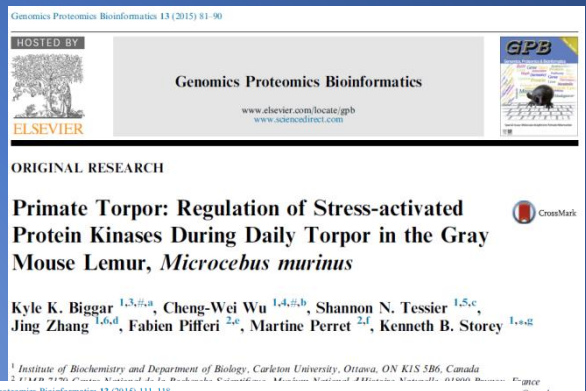
ORIGINAL RESEARCH

Induction of Antioxidant and Heat Shock Protein Responses During Torpor in the Gray Mouse Lemur, *Microcebus murinus*

Cheng-Wei Wu [1,3,#,a], Kyle K. Biggar [1,4,#,b], Jing Zhang [1,5,c], Shannon N. Tessier [1,6,d], Fabien Pifferi [2,e], Martine Perret [2,f], Kenneth B. Storey [1,*,g]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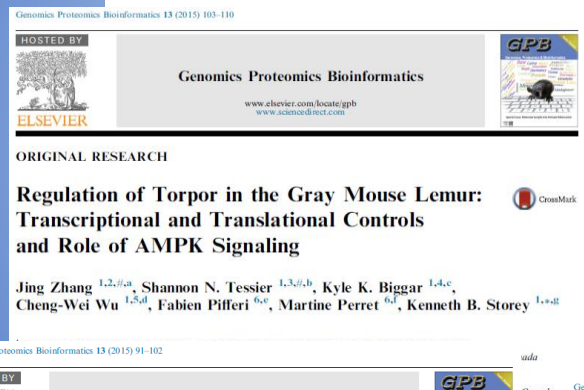
ORIGINAL RESEARCH

Primate Torpor: Regulation of Stress-activated Protein Kinases During Daily Torpor in the Gray Mouse Lemur, *Microcebus murinus*

Kyle K. Biggar [1,3,#,a], Cheng-Wei Wu [1,4,#,b], Shannon N. Tessier [1,5,c], Jing Zhang [1,6,d], Fabien Pifferi [2,e], Martine Perret [2,f], Kenneth B. Storey [1,*,g]

[1] *Institute of Biochemistry and Department of Biology, Carleton University, Ottawa, ON K1S 5B6, Canada*

Regulation of gene/protein expression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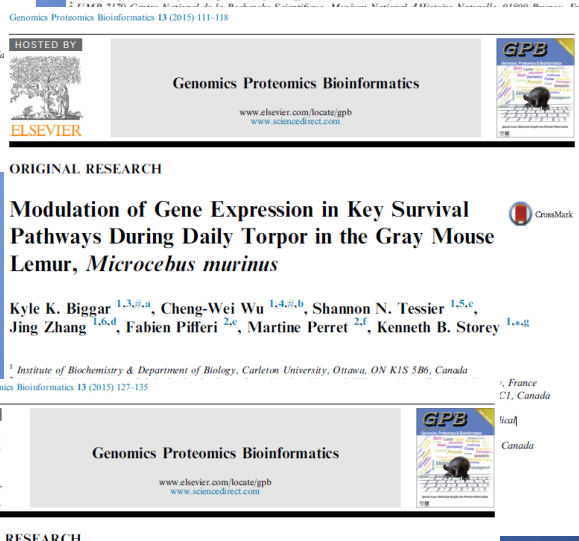
ORIGINAL RESEARCH

Regulation of Torpor in the Gray Mouse Lemur: Transcriptional and Translational Controls and Role of AMPK Signaling

Jing Zhang [1,2,#,a], Shannon N. Tessier [1,3,#,b], Kyle K. Biggar [1,4,c], Cheng-Wei Wu [1,5,d], Fabien Pifferi [6,e], Martine Perret [6,f], Kenneth B. Storey [1,*,g]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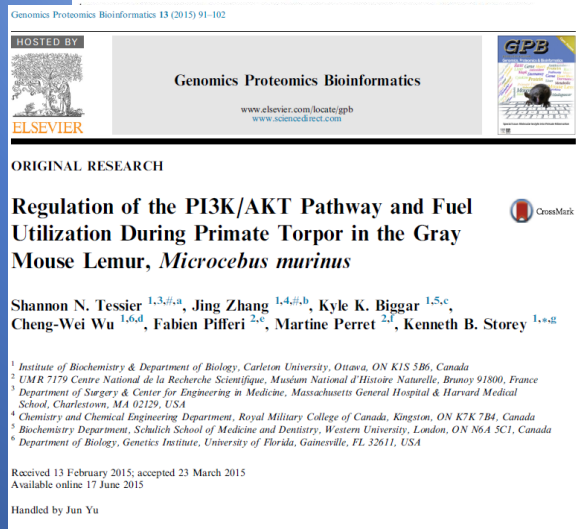
ORIGINAL RESEARCH

Modulation of Gene Expression in Key Survival Pathways During Daily Torpor in the Gray Mouse Lemur, *Microcebus murinus*

Kyle K. Biggar [1,3,#,a], Cheng-Wei Wu [1,4,#,b], Shannon N. Tessier [1,5,c], Jing Zhang [1,6,d], Fabien Pifferi [2,e], Martine Perret [2,f], Kenneth B. Storey [1,*,g]

[1] *Institute of Biochemistry & Department of Biology, Carleton University, Ottawa, ON K1S 5B6, Canada*

Metabolism, fuel utilization, and cytokines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ORIGINAL RESEARCH

Regulation of the PI3K/AKT Pathway and Fuel Utilization During Primate Torpor in the Gray Mouse Lemur, *Microcebus murinus*

Shannon N. Tessier [1,3,#,a], Jing Zhang [1,4,#,b], Kyle K. Biggar [1,5,c], Cheng-Wei Wu [1,6,d], Fabien Pifferi [2,e], Martine Perret [2,f], Kenneth B. Storey [1,*,g]

[1] *Institute of Biochemistry & Department of Biology, Carleton University, Ottawa, ON K1S 5B6, Canada*
[2] *UMR 7179 Centre National de la Recherche Scientifique, Muséum National d'Histoire Naturelle, Brunoy 91800, France*
[3] *Department of Surgery & Center for Engineering in Medicine, Massachusetts General Hospital & Harvard Medical School, Charlestown, MA 02129, USA*
[4] *Chemistry and Chemical Engineering Department, Royal Military College of Canada, Kingston, ON K7K 7B4, Canada*
[5] *Biochemistry Department, Schulich School of Medicine and Dentistry, Western University, London, ON N6A 5C1, Canada*
[6] *Department of Biology, Genetics Institute, University of Florida, Gainesville, FL 32611, USA*

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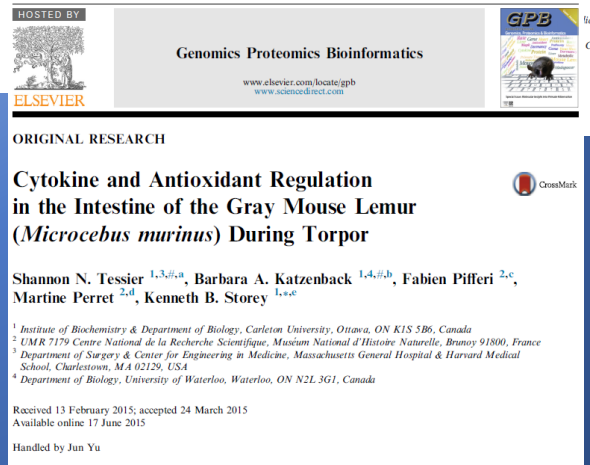
Handled by Jun Yu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ORIGINAL RESEARCH

Cytokine and Antioxidant Regulation in the Intestine of the Gray Mouse Lemur (*Microcebus murinus*) During Torpor

Shannon N. Tessier [1,3,#,a], Barbara A. Katzenback [1,4,#,b], Fabien Pifferi [2,c], Martine Perret [2,d], Kenneth B. Storey [1,*,e]

[1] *Institute of Biochemistry & Department of Biology, Carleton University, Ottawa, ON K1S 5B6, Canada*
[2] *UMR 7179 Centre National de la Recherche Scientifique, Muséum National d'Histoire Naturelle, Brunoy 91800, France*
[3] *Department of Surgery & Center for Engineering in Medicine, Massachusetts General Hospital & Harvard Medical School, Charlestown, MA 02129, USA*
[4] *Department of Biology, University of Waterloo, Waterloo, ON N2L 3G1, Canada*

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TORPOR CONTROL BY SIGNALING CASCADES: Insulin signaling

Luminex: insulin, PI3K/Akt signaling & mTOR protein synthesis pathway

Elements of Insulin/IGF signaling inhibited in muscle & white adipose -- indicates suppression of nutrient-based anabolic /growth responses

Heart showed strong activation of GSK3α indicating a role in cardiac responses

Inhibition of carbohydrate catabolism occurred at PDH in muscle

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ORIGINAL RESEARCH

Regulation of the PI3K/AKT Pathway and Fuel Utilization During Primate Torpor in the Gray Mouse Lemur, *Microcebus murinus*

Shannon N. Tessier [1,3,#,a], Jing Zhang [1,4,#,b], Kyle K. Biggar [1,5,c], Cheng-Wei Wu [1,6,d], Fabien Pifferi [2,e], Martine Perret [2,f], Kenneth B. Storey [1,*,g]

[1] *Institute of Biochemistry & Department of Biology, Carleton University, Ottawa, ON K1S 5B6, Canada*
[2] *UMR 7179 Centre National de la Recherche Scientifique, Muséum National d'Histoire Naturelle, Brunoy 91800, France*
[3] *Department of Surgery & Center for Engineering in Medicine, Massachusetts General Hospital & Harvard Medical School, Charlestown, MA 02129, USA*
[4] *Chemistry and Chemical Engineering Department, Royal Military College of Canada, Kingston, ON K7K 7B4, Canada*
[5] *Biochemistry Department, Schulich School of Medicine and Dentistry, Western University, London, ON N6A 5C1, Canada*
[6] *Department of Biology, Genetics Institute, University of Florida, Gainesville, FL 32611, USA*

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KEYWORDS
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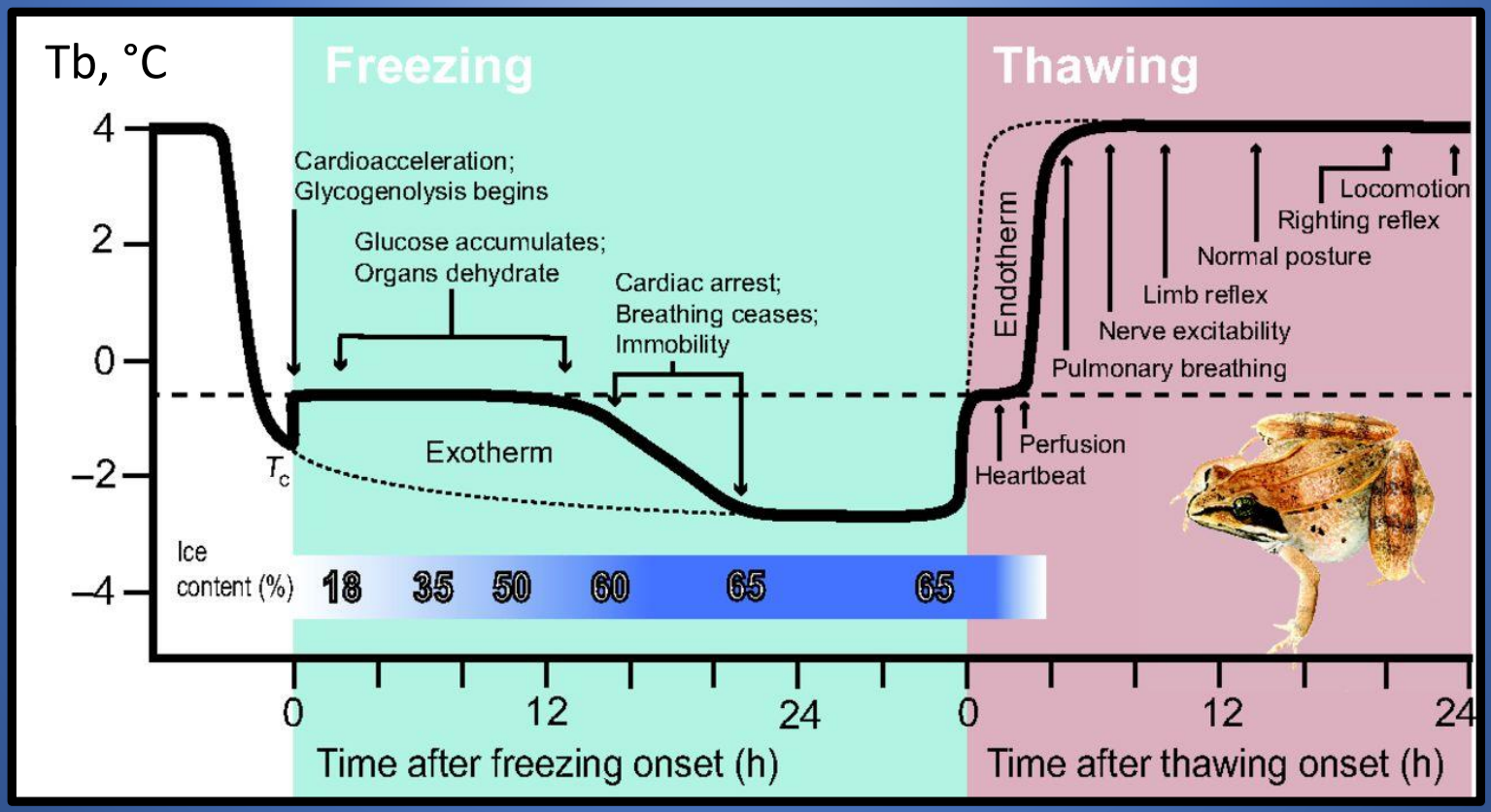
GSK3;
Pyruvate dehydrogenase;
Metabolic rate depression

Abstract Gray mouse lemurs (*Microcebus murinus*) from Madagascar present an excellent model for studies of torpor regulation in a primate species. In the present study, we analyzed the response of the insulin signaling pathway as well as controls on carbohydrate sparing in six different tissues of torpid versus aroused gray mouse lemurs. We found that the relative level of phospho-insulin receptor substrate (IRS-1) was significantly increased in muscle, whereas the level of phospho-insulin receptor (IR) was decreased in white adipose tissue (WAT) of torpid animals, both suggesting an inhibition of insulin/insulin-like growth factor-1 (IGF-1) signaling during torpor in these tissues. By contrast, the level of phospho-IR was increased in the liver. Interestingly, muscle,

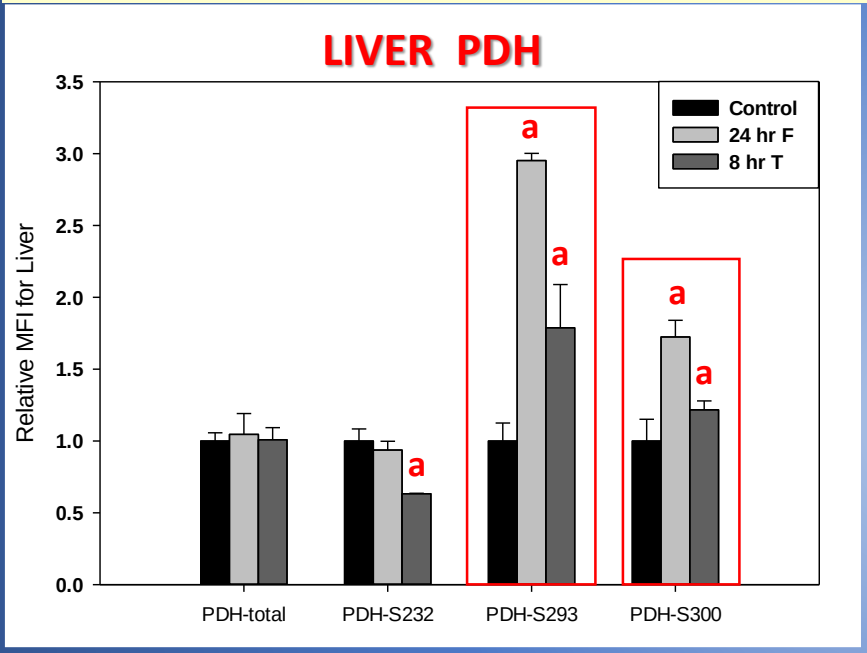
Freeze Tolerance



Costanzo and Lee, 2013

PDH: Responses to Freeze/Thaw

Luminex: Control 5°C, 24 h Frozen -3°C, 8 h Thawed 5°C

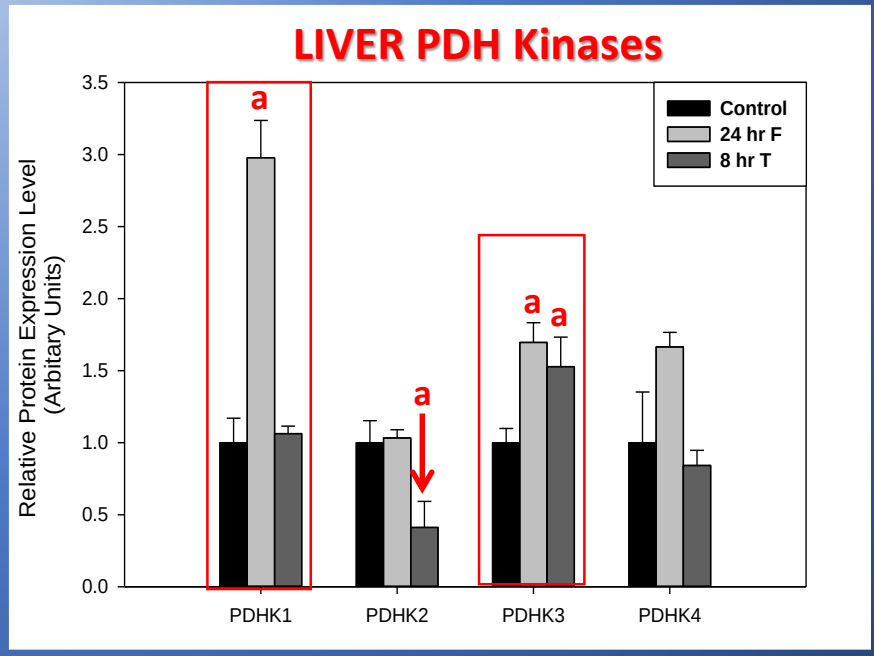


LIVER PDH in Freeze/thaw:

- No change in total PDH protein
- During freezing P-S293 & P-S300 ↑
- During thawing all 3 phospho-sites ↓
- PDH activity is inhibited in the frozen state

LIVER PDH Kinases in Freeze/thaw:

- PDHK1 and PDHK3 ↑ in freezing
- Phosphorylation of PDH ↑
- Corresponds to p-PDH data
- PDH activity is inhibited in the frozen state

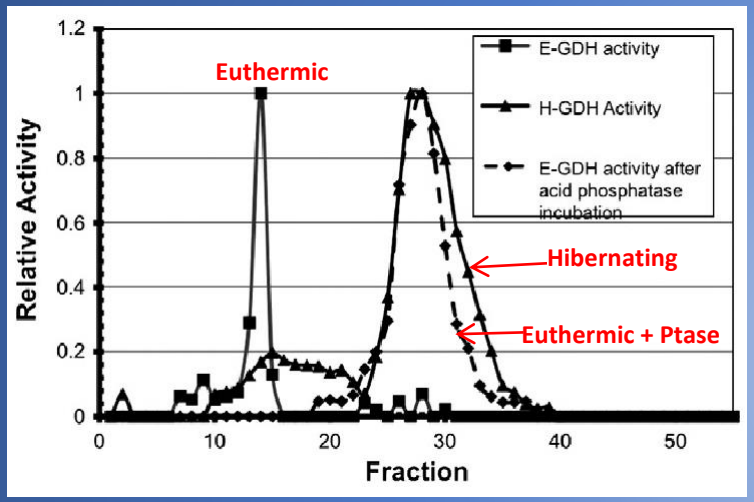


Metabolic Rate Depression & Regulation of Other Mitochondrial Enzymes

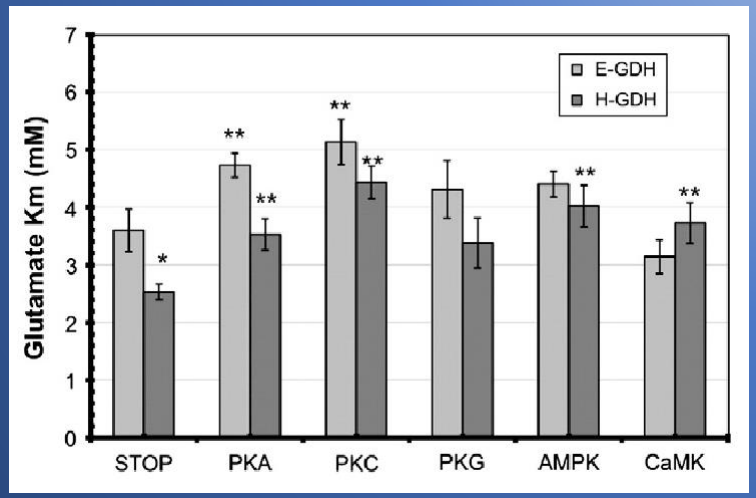
GENERAL PRINCIPLES of reversible transitions:

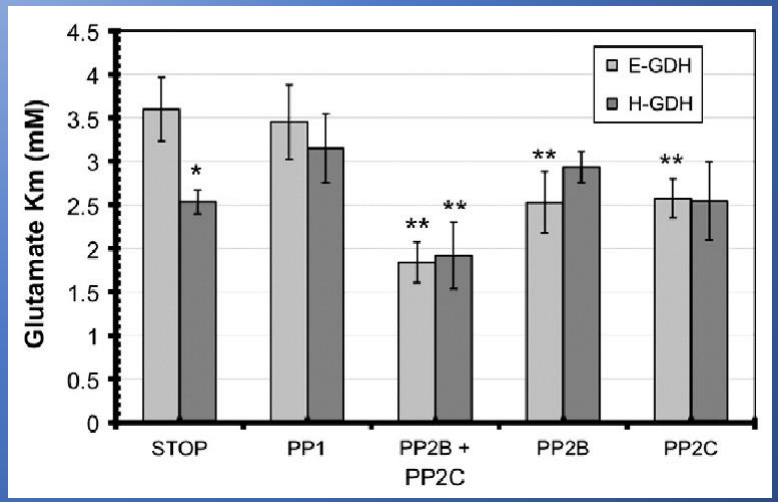
1. A few genes & proteins are specifically altered
2. Most do not require major changes in GENE expression
3. Most do not require major changes in PROTEIN expression
4. REVERSIBLE mechanisms such as POSTTRANSLATIONAL MODIFICATIONS mainly adapt enzyme function
5. Many types – e.g. phosphorylation, acetylation, methylation, etc.
6. Mediate coordinated changes in enzyme & pathway function
7. Mechanisms conserved across phylogeny

Glutamate dehydrogenase in Hibernation



- Two forms of GDH separable on CM cellulose
- Two forms differ in K_m glutamate, V_{max} and activation by ADP
- Phosphatase treatment shifts euthermic form to behave like hibernation form
- Protein kinases have opposite effect





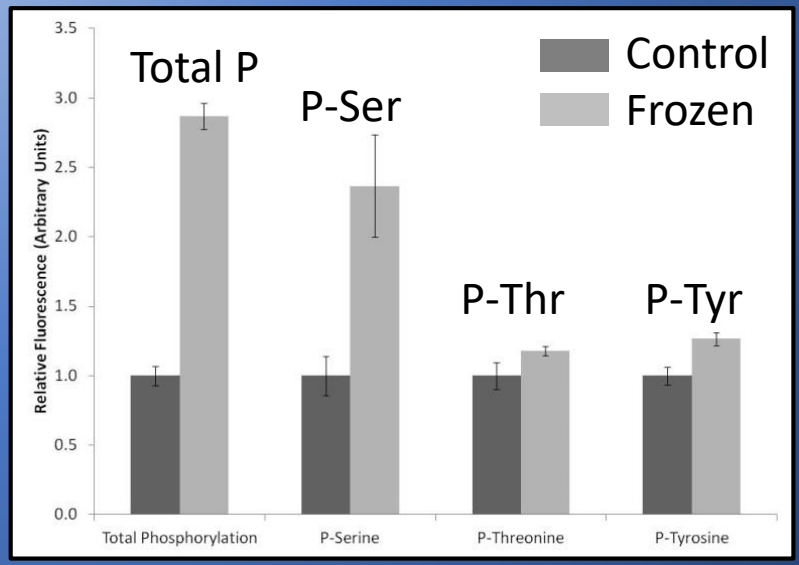
Mn-SOD in Freeze Tolerance

Mitochondrial Mn-SOD Purified from muscle

Enzyme from Frozen Frogs (vs control)

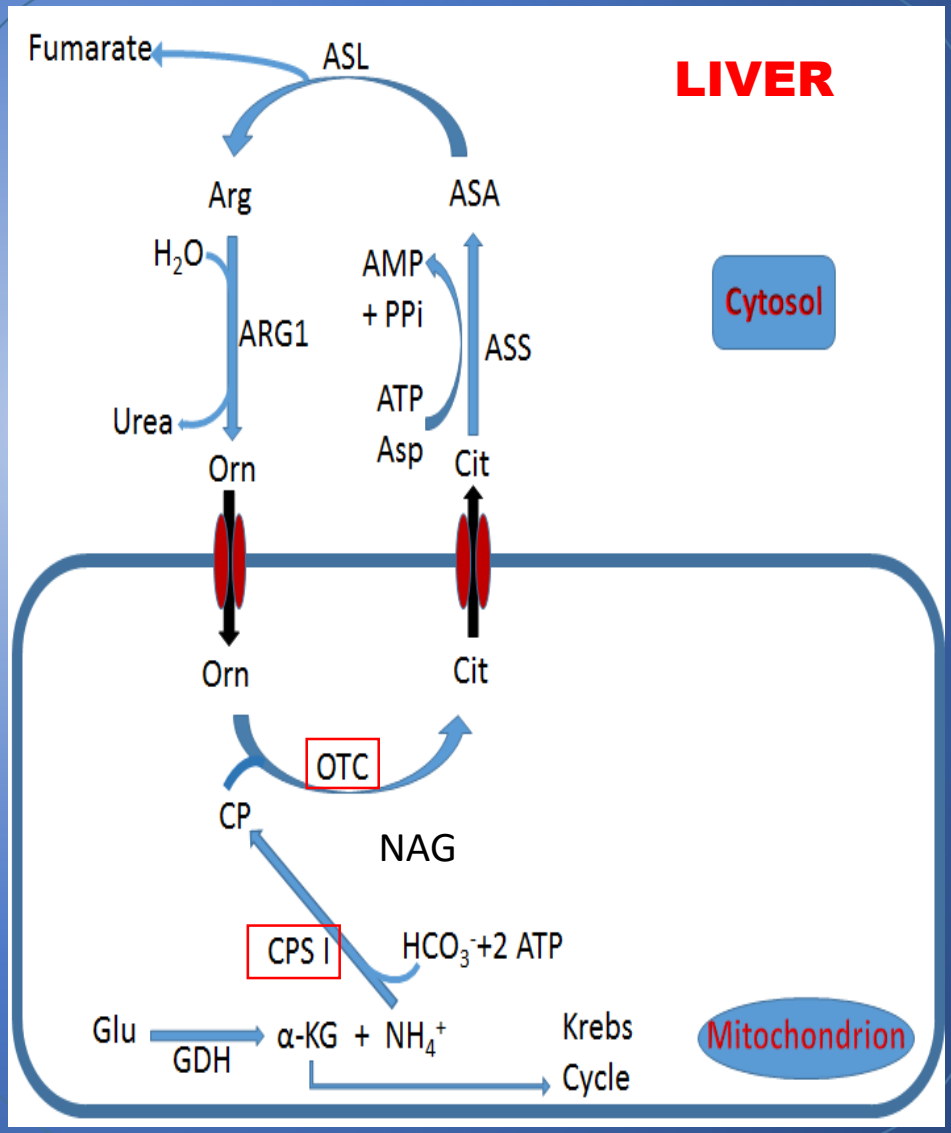
- No change: mRNA or protein levels
- Km xanthine ↓ 34% - higher substrate affinity
- Cm urea ↑ 15% - greater stability

- Phosphorylation state
  Serine-P ↑ 2.4 fold



Urea Cycle & Freeze Tolerance

- Frogs accumulate UREA to defend against dehydration during freezing
- NH_3 is toxic → convert to urea
- Urea cycle mainly in liver
- Involves both mitochondria and cytosol
- Rate limiting step is carbamoyl phosphate synthetase I (CPS1) – activated by N-acetylglutamate
- Ornithine transcarbamylase (OTC) is 2nd step to produce citrulline



CPS1 & OTC Response to Freezing

- Liver OTC from frozen frogs showed:
  - increased affinity for ornithine and carbamoyl phosphate (=lower K_m values)
  - increased serine phosphorylation
- Liver CPS1 from frozen frogs showed:
  - lower K_m for NH_3
  - reduced phosphorylation
  - decreased protein stability (melting temperature)
- Modifications to urea cycle increase affinity for substrates

OTC

Ornithine Affinity	↑
Carbamoyl Phosphate Affinity	↑
Serine Phosphorylation	↑

CPS1

Ammonia Affinity	↑
Phosphorylation	↓
Thermal stability (T_m)	↓

PRINCIPLES OF HIBERNATION

1. Metabolic rate reduction
2. Control by protein kinases
   (SAPKs, 2nd messenger PKs)

[3. Most Genes OFF]

4. Selective gene activation

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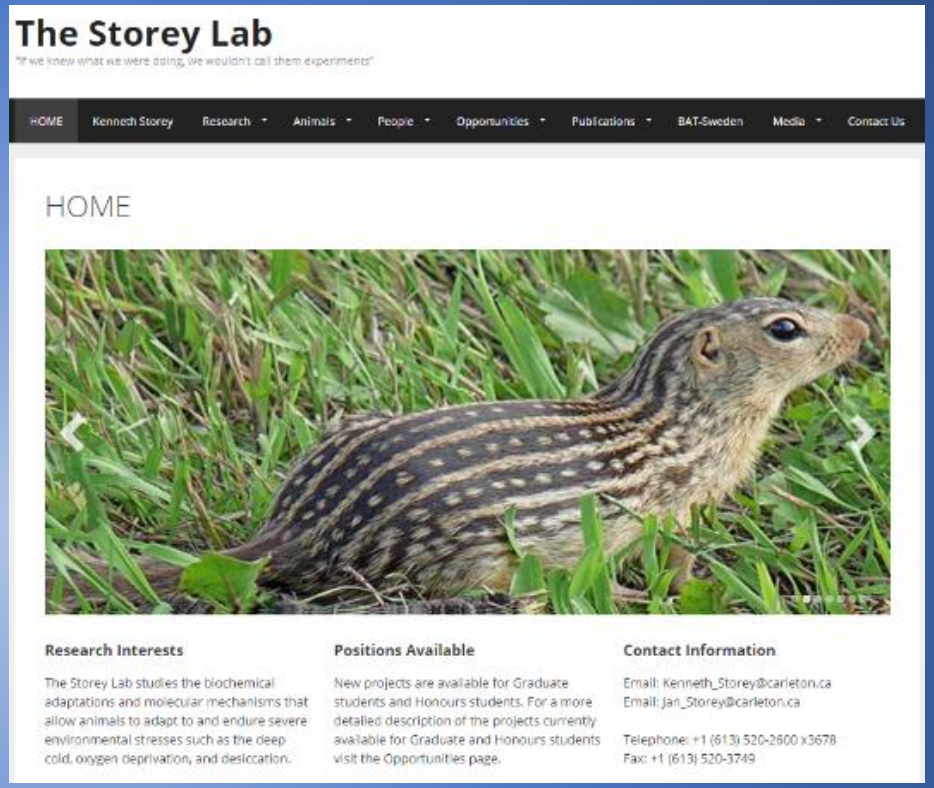

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MAMMALIAN HIBERNATION

- Key characteristics :
  - –metabolic rate depression (hypometabolism)
  - –low body temperatures
  - –Hibernation is a NATURAL model system

- Purpose is to overcome food shortages and the high energy costs of endothermy (warm-blooded)