# Phosphorylation status of pyruvate dehydrogenase in the mousebird *Colius striatus* undergoing torpor

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## Abstract

Torpor is a heterothermic response that occurs in some animals to reduce metabolic expenditure. The speckled mousebird (*Colius striatus*) belongs to one of the few avian taxa possessing the capacity for pronounced torpor, entering a hypometabolic state with concomitant decreases in body temperature in response to reduced food access or elevated thermoregulatory energy requirements. The pyruvate dehydrogenase complex (PDC) is a crucial site regulating metabolism by bridging glycolysis and the Krebs cycle. Three highly conserved phosphorylation sites are found within the E1 enzyme of the complex that inhibit PDC activity and reduce the flow of carbohydrate substrates into the mitochondria. The current study demonstrates a marked increase in S232 phosphorylation during torpor in liver, heart, and skeletal muscle of *C. striatus*. The increase in S232 phosphorylation during torpor was particularly notable in skeletal muscle where levels were ~49-fold higher in torpid birds compared to controls. This was in contrast to the other two phosphorylation sites (S293 and S300) which remained consistently phosphorylated regardless of tissue. The relevant PDH kinase (PDHK1) known to phosphorylate S232 was found to be substantially upregulated (~5-fold change) in the muscle during torpor as well as increasing moderately in the liver (~2.2-fold increase). Additionally, in the heart, a slight (~23%) decrease in total PDH levels was noted. Taken together the phosphorylation changes in PDH suggest that inhibition of the complex is a common feature across several tissues in the mousebird during torpor and that this regulation is mediated at a specific residue.

**Keywords**: mousebird, protein phosphorylation, pyruvate dehydrogenase, metabolism, torpor

## Highlights

- Increased pyruvate dehydrogenase kinase 1 in torpor mousebird muscle and liver.
- Greater phosphorylation of PDH S232 in muscle, liver, and heart in torpid mousebirds.
- Reduction in total PDH in heart tissue from birds in torpor.

## 1 INTRODUCTION

The ability to maintain a constant body temperature optimal for biochemical and physiological processes is a central feature of both mammalian and avian thermal physiology. Birds maintain a constant internal body temperature of around 40°C through physiological means, allowing them to remain active under a wide variety of environmental conditions whereas ectothermic species must rely on behavioral responses to alter their body temperature (Prinzinger et al., 1991). While physiological homeostasis of body temperature benefits animals by enabling them to live a more active lifestyle, maintaining an elevated body temperature can become disadvantageous when the ambient temperature is low and/or food supplies are limited due to the high energetic costs associated with homeothermy. For this reason, many small endotherms employ heterothermic strategies to minimize metabolic losses during periods of inactivity ranging from seasonal (hibernation) to daily (torpor) time frames. Daily torpor involves a reduction in internal body temperature during the inactive phase of the circadian cycle. While daily heterothermic responses are quite common amongst small mammals, they are comparatively rare among birds, with the capacity for pronounced torpor restricted to a subset of taxa (Ruf & Geiser, 2015).

The mousebirds (Coliiformes) are an avian order endemic to Africa. They allow their body temperature to drop and enter torpor at night to minimize fuel consumption during periods of reduced caloric intake or when deprived of the thermoregulatory benefits of communal roosting 2001b. The speckled mousebird (*Colius striatus*) is the best-studied species with respect to torpor responses. These birds typically roost communally with conspecifics to conserve body heat, but if that fails or is not possible they enter a heterothermic state where body temperature can fall to as low as 18.2°C (Brown & Foster, 1992; McKechnie & Lovegrove, 2001a, 2001b; McKechnie et al., 2006). Whereas there have been a variety of studies investigating the physiology of mousebird torpor, the underlying biochemistry of the phenomenon has only recently begun to receive attention. A recent study by the current authors demonstrated that the Akt-mTOR signaling pathway is regulated in a tissue-specific manner in *C. striatus* during torpor to restrict energetically expensive metabolic processes such as protein translation (Green et al., 2020). A crucial locus in fuel metabolism is the mitochondrial pyruvate dehydrogenase complex (PDC) reaction that gates the irreversible aerobic catabolism of carbohydrate fuels (e.g., glycogen, glucose) by the mitochondria. This enzyme is closely regulated during mammalian hibernation to minimize consumption of carbohydrate fuels and instead promote stored lipid reserves as the primary fuel (Wijenayake et al., 2017). The current study provides the first investigation of torpor-responsive PDC regulation in birds to achieve carbohydrate sparing in the hypometabolic state.

The PDC is a large mitochondrial enzyme complex whose regulation constitutes a crucial checkpoint of central energy-producing reactions in cells. The PDC serves as a bridge linking glycolysis to the tricarboxylic acid (TCA) cycle by decarboxylation and oxidation of pyruvate to generate $CO_2$ and acetyl-CoA, the latter being integrated into the TCA cycle via the ligation to oxaloacetate to generate citrate by citrate synthase. The reaction mediated by the PDC is highly favorable ($\Delta G = -39.26$ kJ/mol) and as such, is essentially irreversible in the cell making

it an ideal point of regulation (Li et al., 2011). The PDC is composed of three distinct but physically associated enzymes that carry out the role of oxidizing pyruvate: (a) pyruvate dehydrogenase (PDH or E1), (b) dihydrolipoamide acetyltransferase (DLAT or E2), and (c) dihydrolipoamide dehydrogenase (DLD or E3). Each complex contains many copies of each of these proteins resulting in a molecular weight of an intact complex of about 6.1 MDa in *Escherichia coli* (Danson et al., 1979). Whereas each of the subunits play critical roles in PDC function, control of the activity of the E1 or PDH subunit is generally thought to be the rate-limiting step in the overall rate of conversion of pyruvate to acetyl-CoA.

Control over the PDC is maintained by the presence or absence of phosphate groups ligated to key serine residues on the E1α subunit. These residues are highly conserved in vertebrates and in the human E1α homolog correspond to S232, S293, and S300. Phosphorylation at these hydroxyl-bearing residues is associated with a pronounced decrease in PDC activity and serves as an important regulatory site for the overall state of carbohydrate oxidation in the cell (Hadj-Moussa et al., 2018). Activity of the PDC is indirectly related to the availability of the divalent metal cations $Ca^{2+}$, and $Mg^{2+}$ within the mitochondrial matrix as these ions are important in facilitating the recruitment of pyruvate dehydrogenase phosphatase (PDP) to the complex and thereby promoting the removal of phosphate groups to restore functionality of the complex (Lander et al., 2018; Thomas et al., 1986). Conversely high levels of mitochondrial ATP, an indicator of ample fuel supplies, promotes the activity of the pyruvate dehydrogenase kinases (PDKs 1–4) that phosphorylate and reduce PDH activity (Sugden & Holness, 2003).

## 2 MATERIALS AND METHODS

### 2.1 Animal treatments

Speckled mousebirds, 10 animals total, were captured near Pretoria, South Africa, using baited walk-in traps as described previously (Green et al., 2020). Birds were housed and assigned to either a control euthermic group or a heterothermic torpor group (five in each group) at the University of Pretoria Experimental Farm. Birds were fed ad libitum until the start of experimentation at which point each individual had a passive integrated transponder tag injected into their abdominal cavities to monitor their body temperature. Mousebirds in the torpid group were withheld food from 09:00 onwards during the day for a total of three consecutive days with an ambient temperature of 10°C, whereas the control group was kept at an ambient temperature of 30°C. All five birds in the torpor group were confirmed to be in a torpid state by having an internal body temperature <20°C and an absence of responses to external stimuli, whereas the euthermic birds had a body temperature greater than 35°C and normal responses and levels of activity. All experimental procedures were approved in advance by the University of Pretoria's Animal Ethics Committee (protocol EC051-16) and the Research Ethics and Scientific Committee of the South African National Biodiversity Institute (P16/24) (Green et al., 2020). Following euthanasia by cervical dislocation as soon as internal body temperature had dropped below 20°C in the torpor group, tissues were rapidly dissected from the birds and flash-frozen in liquid nitrogen. Tissues were shipped on dry ice to Carleton University (Ottawa, Canada) where they were held at −72°C until use.

### 2.2 Protein extract preparation

Homogenization of the tissue samples (liver, skeletal [pectoral] muscle, heart, brain) from four randomly selected animals in each group was carried out according to previously described protocols using the 1× lysis buffer provided with the Luminex® assay kits with the addition of

1 mM $Na_3VO_4$, 10 mM NaF, 10 mM β-glycerophosphate, 10 µl/ml of protease inhibitor cocktail (BioShop, # PIC002.1) to ensure protein stability and retention of the original phosphorylation state (Green et al., 2020). Samples were then sonicated for 15 s and incubated on ice for 30 min with intermittent mixing every 10 min. Samples were subsequently centrifuged at 14,000*g* for 20 min at 4°C and the supernatant was decanted and stored at −80°C until use.

### 2.3 Multiplex assay

Relative levels of different phosphorylated residues (S232, S293, S300) and the total amount of PDH in the mousebird samples were quantified using the Multi-Species Pyruvate Dehydrogenase Complex Magnetic Bead Panel (EMD Millipore; PDHMAG13K) assay kit and were read with a Luminex 100® machine. The manufacturer's protocols were followed as described in a previous paper (Green et al., 2020). The Luminex® assay kit makes use of fluorescent color-coded beads that are bound to antibodies that recognize a phosphorylation site of interest. A second antibody recognizes bound antigen and itself is conjugated with biotin that can be detected through a streptavidin-phycoerythrin conjugate. Beads are read using a two-laser system, the first identifies the phosphorylation site of interest by determining the identity of the bead being read and the second determines the signal intensity by detecting phycoerythrin.

### 2.4 Western blots

Western blots were performed for PDHK1 quantification since this is the only known kinase targeting the S232 residue which was found to have elevated phosphorylation levels in three of the four tissues examined in the multiplex assay. Western blots used 25 µg of protein for each sample loaded into a 10% SDS-PAGE electrophoresis gel and run at 180 V for 65 min. Proteins were transferred to a PVDF membrane according to previously described methods and subsequently blocked with 2% w:v skim milk for 30 min (Green et al., 2020). After removing the milk solution the blots were washed 3 × 5 min each time with TBST (20 mM Tris base, 140 mM NaCl, pH 7.6, 0.05% v:v Tween-20) and the blots were incubated overnight in 1:1000 diluted anti-PDHK antibody (GeneTex, GTX107405). The blots were washed again for 3 × 5 min and probed with an HRP-conjugated anti-rabbit secondary diluted 1:8000 v:v. Blots were washed with TBST 3 × 5 min again and then visualized using an enhanced chemiluminescent (ECL) protocol. Blots were stained with Coomassie brilliant blue and the signal from a group of stably expressed protein bands in the Coomassie-stained image were used as the loading control (Eaton et al., 2013). Blots were quantified using Gene Tools 4.3.8.0 software using manual background correction for band intensity by comparison to a nearby unoccupied area on the membrane.

### 2.5 Multiple sequence alignments

A partial sequence of the speckled mousebird PDH E1 alpha subunit is known (NCBI accession # XP_010194171.1) (G. Zhang et al., 2014) and was aligned using the Clustal Omega multiple sequence alignment tool (Sievers et al., 2011) to the human sequence (NCBI accession # NP_000275.1) to demonstrate that all the phosphorylation sites probed were conserved in the mousebird protein. These two sequences were also compared to other species to show the degree of conservation between different vertebrates: African clawed frog (*Xenopus laevis*: accession # NP_001086638.1), zebrafish (*Danio rerio*: accession # XP_005162743.1) as well as an invertebrate species (*Drosophila melanogaster*: accession # NP_572181.4).

## 2.6 Statistics

Data obtained from the Luminex® assays were standardized to the respective control euthermic group. Data are the means of four independent biological replicates from different animals ±SEM and torpor values are considered significantly differenct from the corresponding control euthermic values through evaluation by the Student's *t* test with $p < 0.05$. Creation of bar charts and statistical evaluations were performed using RBioplot software (J. Zhang & Storey, 2016).

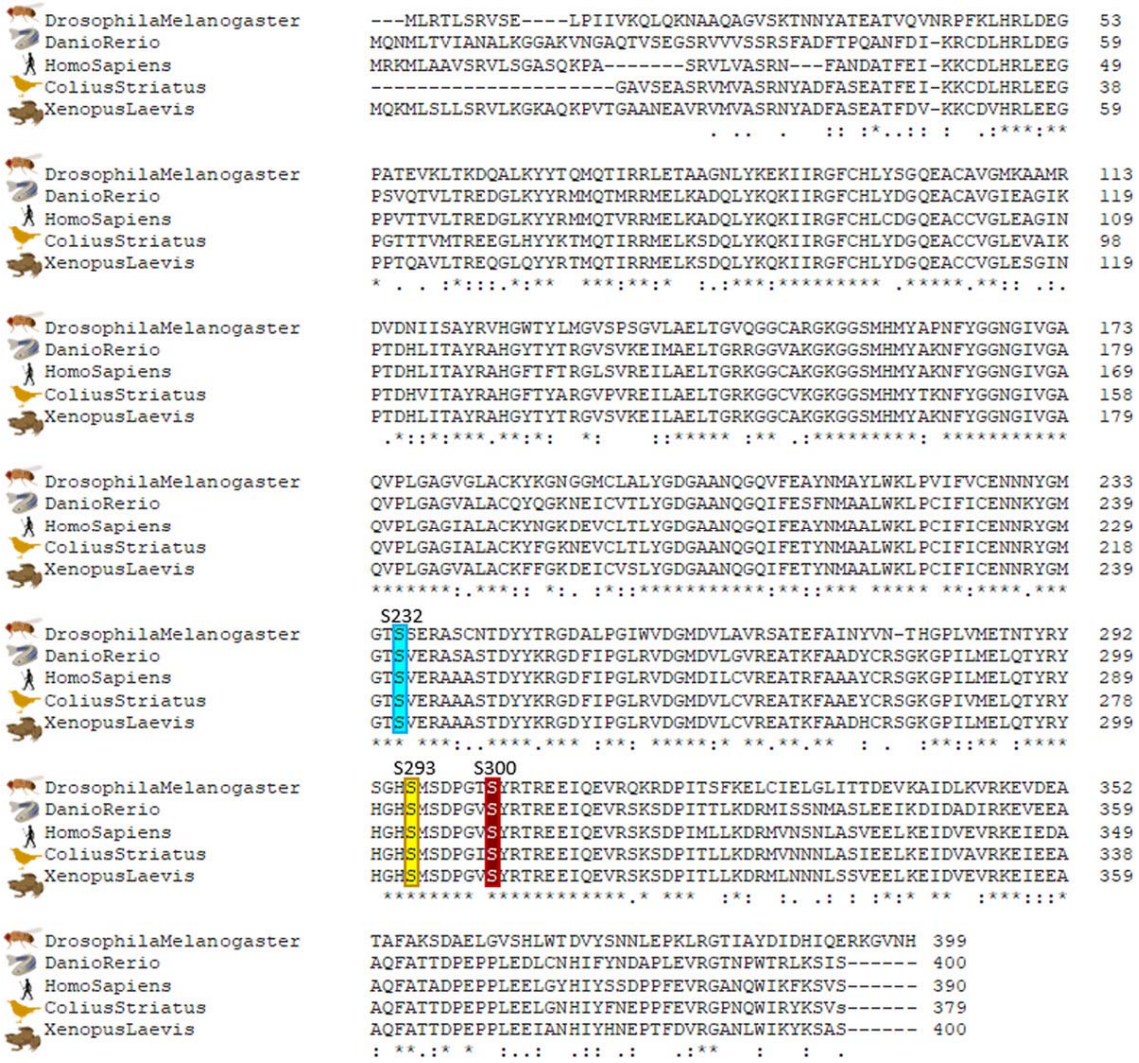


**Figure 1.** Protein sequence alignment of partial *Colius striatus* (speckled mousebird) PDH sequence compared to the African clawed frog (*Xenopus laevis*), human (*Homo sapiens*), zebra fish (*Danio rerio*), and the common fruit fly (*Drosophila melanogaster*). The three serine residues known to have a regulatory role in the function of PDH are highlighted and labeled and show conservation across all species in the alignment. "*" represents an amino acid that is conserved in all sequences; ":" represents residues that share strongly similar properties; "." residues have more weakly similar properties. PDH, pyruvate dehydrogenase

## 3 RESULTS

Alignment of the human PDH sequence to an available partial mousebird sequence demonstrated a high degree of similarity (88.11% identity). Importantly for the purposes of this study, all the known serine phosphorylation sites that have strong regulatory significance in the

human sequence were conserved in the aligned mousebird sequence (Figure 1). Indeed, this sequence appears to be highly conserved in all vertebrates as two additional sequence alignments showed these same serine residues to be conserved in a frog species (*X. laevis* accession # NP_001086638) and a fish species (*D. rerio* accession # XP_005162743). These critical regulatory serines were also conserved within the *D. melanogaster* homolog of the protein (accession # NP_572181.4).

Luminex multiplex assays were carried out with homogenates of four tissues from mousebirds to assess the degree of phosphorylation of the three regulatory phosphorylation sites on PDH and the total levels of PDH. In the liver, the level of phosphorylation remained consistent across the sites homologous to S293 and S300, but S232 displayed a considerably higher level of phosphorylation (3.71-fold increase) in the torpid state relative to the control (Figure 2). Total levels of PDH remained consistent between the two states.

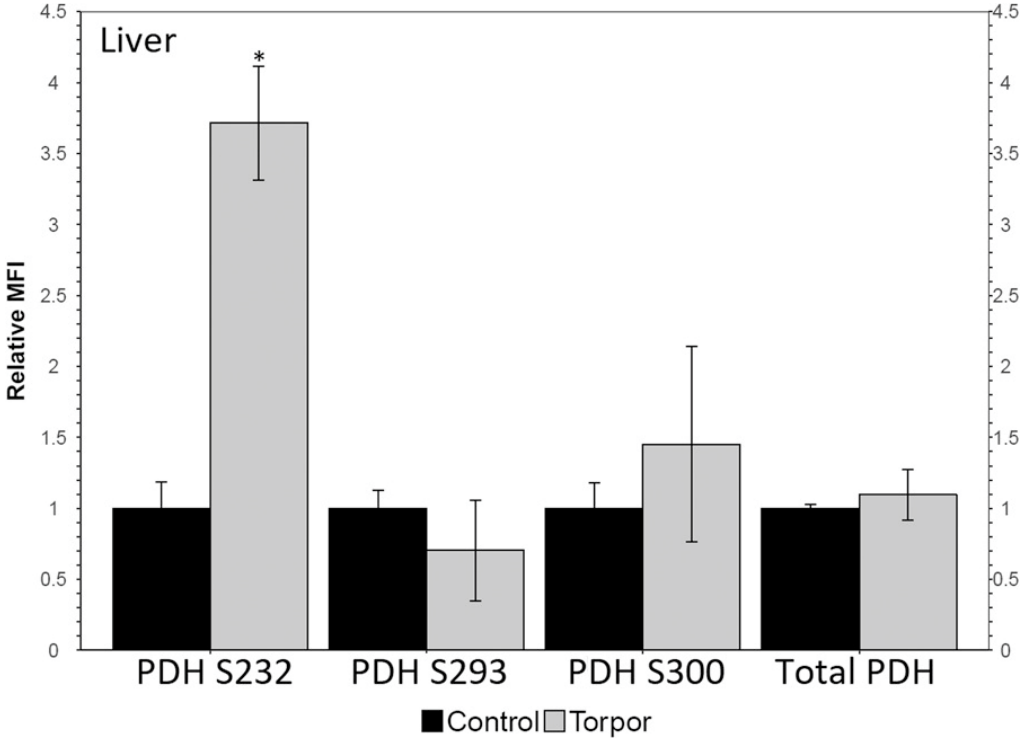


**Figure 2**. Relative phosphorylation for specific regulatory serine sites and total PDH protein in *Colius striatus* liver between control euthermic and torpor conditions. Serine phosphorylation sites are designated by the position of the corresponding homologous serine residue in the human PDH sequence. Protein phosphorylation and total PDH are reported relative to the control for each target with the control standardized to a value of 1. Data represent the mean ± standard error of the mean, $n = 4$ independent tissue samples from separate animals. "*" indicates that the torpor value for the parameter is statistically different from the control ($p < 0.05$ Student's $t$ test). PDH, pyruvate dehydrogenase

Skeletal muscle tissue displayed the most prominent differences between the control and torpid birds. Here a massive 49.2-fold increase was seen between the relative levels of phosphorylation on the S232 residue compared to control euthermic birds (Figure 3). In spite of this large increase, no other changes were seen in the other two phosphorylation sites in the torpor birds or the total levels of PDH.

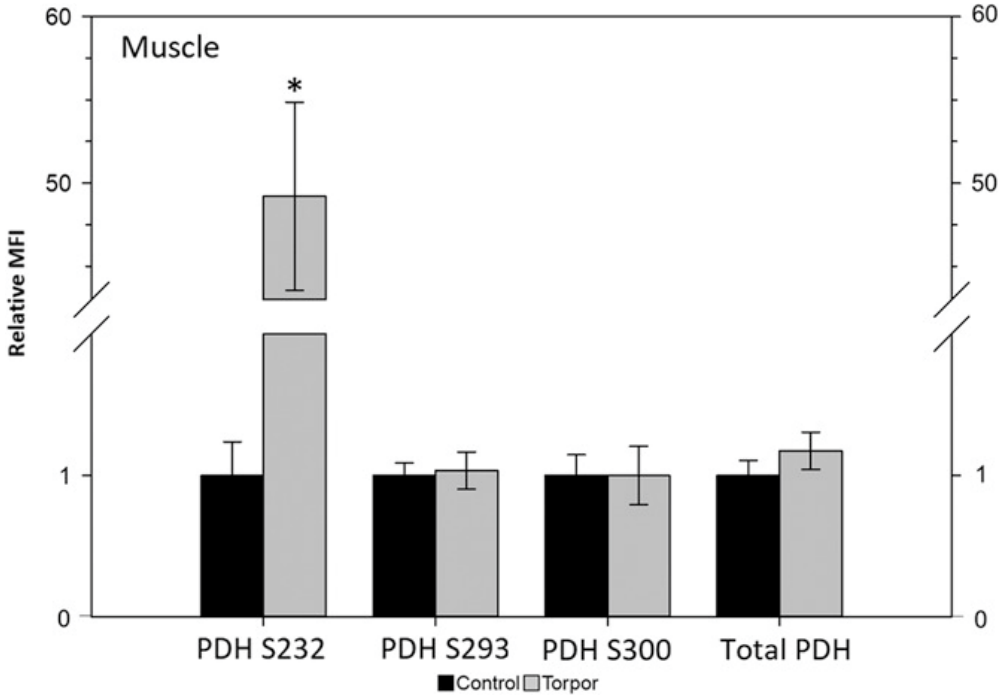


**Figure 3**. Relative phosphorylation for specific regulatory serine sites and total PDH in *Colius striatus* skeletal muscle tissue between control and torpor conditions. Other information as in Figure 2. PDH, pyruvate dehydrogenase

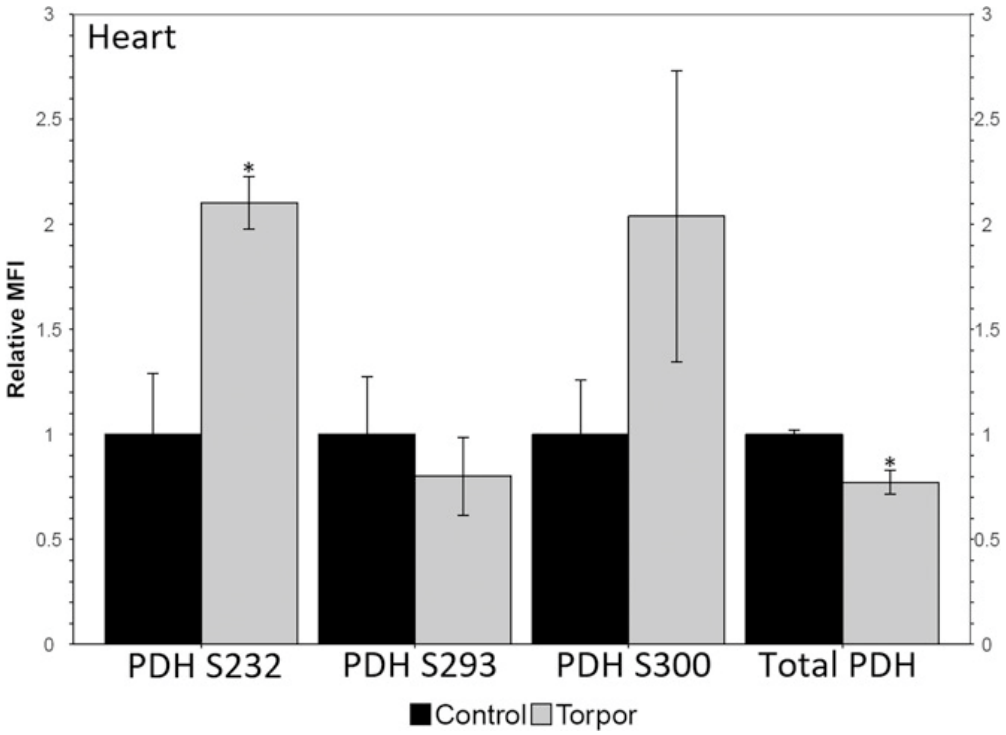


**Figure 4**. Relative phosphorylation for specific regulatory serine sites and total PDH in *Colius striatus* heart tissue between control and torpor conditions. Other information as in Figure 2. PDH, pyruvate dehydrogenase

The hearts of mousebirds also exhibited a significant increase in the relative level of S232 phosphorylation in torpor (2.1-fold increase). Neither of the other two phosphorylation sites were significantly affected during torpor, but a moderate decrease in the level of total PDH was observed in response to torpor (a decrease to 77.3% of euthermic values) (Figure 4).

Analysis of brain tissue from mousebirds did not reveal any significant changes in the four parameters measured in response to torpor. However, the mean value for S232 phosphorylation during torpor was 3.5-fold higher than in the control (Figure 5) but the very large inter-sample variability in S232 phosphorylation in the brain samples from torpid birds obscured any possible significant change in the data ($p = 0.19$ by Student's $t$ test).

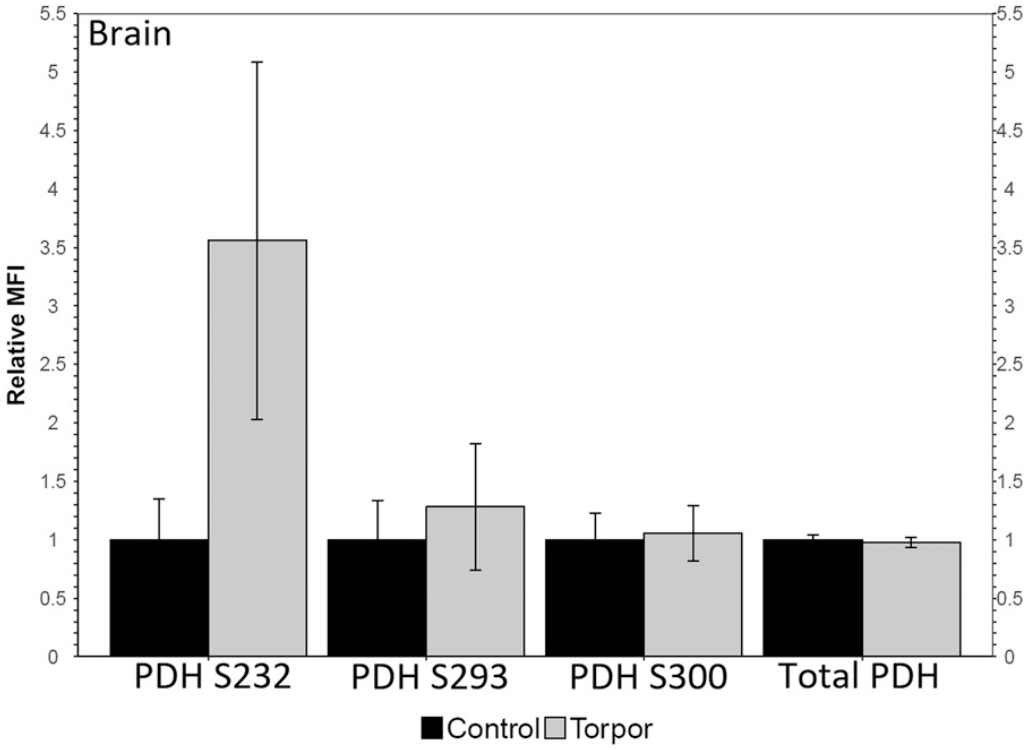


**Figure 5**. Relative phosphorylation for specific regulatory serine sites and total PDH in *Colius striatus* brain between control and torpor conditions. Other information as in Figure 2. PDH, pyruvate dehydrogenase

Western blots for PDHK1 levels (the protein kinase known to be responsible for S232 phosphorylation) showed significant upregulation in two of the three tissues shown to have significantly higher phosphorylation of S232 in the torpid state. Liver samples showed a ~2.2-fold increase in PDHK1 levels compared with euthermic controls and PDHK1 skeletal muscle showed an even greater (~5-fold) increase during torpor (Figure 6).

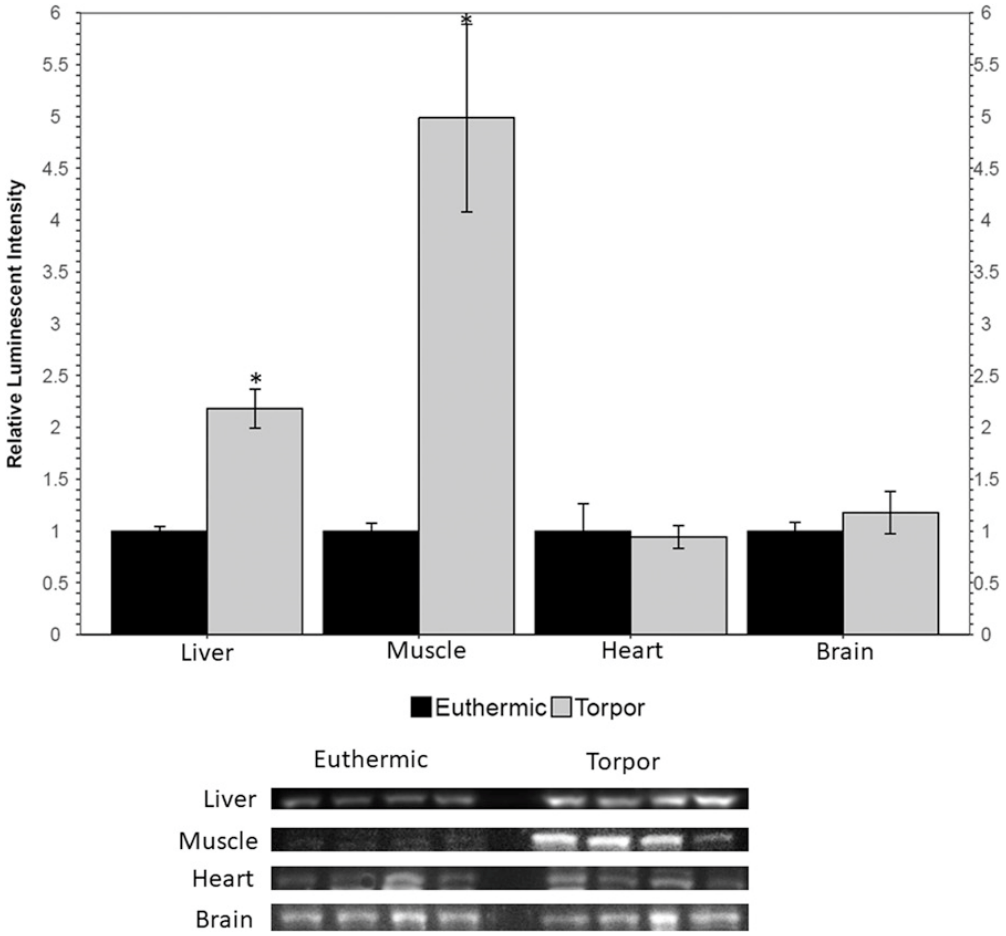


**Figure 6**. Results of western blot analysis experiments demonstrating the relative levels of protein expression of PDHK1 in the mousebird samples. Histogram demonstrates the chemiluminescent signal relative to the control set to 1 and normalized to Coomassie blue staining of a group of proteins that showed constant expression during torpor to correct for any minor differences in loading the gel. All data are $n = 4$ independent samples from separate animals. "*" indicates the average level of PDHK1 in the torpid animals was statistically different from the corresponding euthermic control value for the given tissue.

## 4 DISCUSSION

In animals undergoing metabolic rate depression, such as during torpor or hibernation, control over the central metabolic processes responsible for maintaining energy production is essential to ensure that metabolic fuel reserves are conserved. PDH is a key regulatory locus that serves to regulate the catabolism of carbohydrate fuels, typically in a way that spares limited reserves of carbohydrates in favor of much larger pools of stored triglycerides. Indeed, inhibition of carbohydrate oxidation at the PDH locus is likely key to regulating the switch to a primary reliance on lipid oxidation to fuel hibernation in mammals (Wijenayake et al., 2017). Therefore, PDH is tightly regulated by reversible phosphorylation at three highly conserved serine residues; in the human sequence, these are S232, S293, and S300. Phosphorylation at any of these residues is associated with a pronounced decrease in activity of the whole PDC, thereby reducing the availability of carbohydrate intermediates for oxidation by the Krebs cycle. This contributes to both slowing cellular metabolism and shifting metabolism over to a primary reliance on lipid oxidation.

Sequence alignments were carried out to demonstrate that the mousebird PDH sequence shows conservation of the key serine regulatory sites that are associated with regulation of the complex in mammals and other animals. Fortunately, while only a partial sequence was available for *C. striatus* PDH, the unsequenced portion was in the N-terminal region, which is distant from the area of the protein that contains the serine phosphorylation sites. Performing a sequence alignment demonstrated that *C. striatus* shares 88.11% identity with respect to human PDH and is highly conserved in the regions bearing the serine phosphorylation sites (Figure 1). This high degree of sequence similarity was crucial to the reliability of the assays as it ensured that the antibodies used to evaluate the PDH phosphorylation sites would recognize their targets even though they were originally designed for the human homolog. Indeed, comparisons to two other vertebrate and to *D. melanogaster* PDH sequences demonstrated that these residues are highly conserved, emphasizing the importance of reversible phosphorylation of these serine residues in the regulation of PDH. Inhibition of the PDC via phosphorylation has even been demonstrated in fungi and plants, making this mechanism an almost universal means of metabolic control (Gey et al., 2008; Weraduwage et al., 2016). This demonstrates that the multiplex assay used in this study should have a high degree of cross-species reactivity owing to the highly conserved nature of the amino acid sequences around each of the phosphoserine sites. Previously published studies in our lab have utilized this same multiplex assay in a variety of vertebrate species, including the wood frog (*Rana sylvatica*) and the 13-lined ground squirrel (*Ictidomys tridecemlineatus*), and have both demonstrated excellent sensitivity regardless of species (Al-attar et al., 2019; Wijenayake et al., 2017).

The main observation in this study is the large increase in phosphorylation on the S232 residue in all tissues except the brain. None of the other residues in the torpid tissues were found to be differentially phosphorylated during bouts of torpor, strongly suggesting that any regulatory mechanism controlling metabolic rate must occur at the S232 residue. The massive 49.2-fold increase in phosphorylation at S232 in muscle tissue strongly suggests that some form of regulation is occurring at this locus. This large increase in phosphorylation of muscle PDH at this residue was particularly remarkable owing to the lack of change in the other phosphorylation sites. In most animals undergoing caloric restriction (but not torpor), increases in phosphorylation are expected on all of the serine sites investigated as has been observed previously (Gudiksen & Pilegaard, 2017), thereby suggesting the exclusive increases in S232 phosphorylation observed here during mousebird torpor are important adaptations specifically related to the heterothermic responses in this species. Torpor in mousebirds is a natural extension of caloric restriction, since in this species caloric restriction alone is a sufficient trigger for heterothermic responses (McKechnie & Lovegrove, 2001a), so it is impossible to treat caloric restriction as a separate stress from heterothermic responses, but the observation that only one specific phosphorylation site is upregulated in the experimental group suggests a novel response in this species. A likely interpretation of this observation is that only a subset of PDHKs are responsive for the cellular changes that occur during torpor in this species. Increases in S232 phosphorylation were demonstrated during torpor in a small marsupial species, *Dromiciops gliroides*. This study also demonstrated several increases in phosphorylation at the other sites, however, S232 is notable as it had increased phosphorylation in all tissues tested with the exception of the liver and was the only site to be differentially phosphorylated in the kidney and the skeletal muscle during torpor (Wijenayake et al., 2018). While torpor likely differs greatly between mammalian and avian taxa such as the mousebirds, the importance of S232 phosphorylation in torpor appears to have a wide taxonomic distribution. Of the 4 isoforms of PDHKs, only PDHK1 has been shown to use the S232 site as a substrate for phosphorylation and this particular kinase tends to exhibit low affinity for the other two phosphorylation sites (Korotchkina & Patel, 2001b).

Owing to the significance of PDHK1 in phosphorylating S232 in human PDH E1, the relative expression of this kinase was investigated in the four tissues assessed previously for PDH phosphorylation. As was expected, a considerable increase in the relative level of PDHK1 was observed in response to torpor in mousebird muscle tissue (~5-fold). This reflected the enormous 49.2-fold increase in phosphorylation of S232 in muscle of torpid birds. The liver also showed an increase in levels of PDHK1, albeit to a lesser degree, reflecting the fact that the relative level of phosphorylation was not nearly as great in this tissue (3.71-fold increase). The lack of significant change in PDHK1 expression in the other two tissues was not as surprising considering that the levels of S232 phosphorylation in brain were unchanged and heart displayed the lowest significant increase in phosphorylation of that residue. While the relative tissue distribution of the PDHKs in avian species is unknown, it is known in mammalian models that PDHK1 has a wide tissue distribution, supporting the idea that a universal response in PDH phosphorylation status could be mediated by upstream factors controlling the expression of PDHK1 (Klyuyeva et al., 2019). Taken together, the data presented in the immunoblotting experiments support the observations made with the multiplex data by demonstrating that increases in phosphorylation at S232 correlate well with the change in expression of PDHK1 during torpor.

Overexpression of PDHK1 is thought to be an important mechanism by which cancer cells can exploit the Warburg effect by promoting anaerobic metabolism via inhibition of PDH activity (Saunier et al., 2017). Interestingly, S232 phosphorylation is also thought to be related to decreased stability of the PDH complex and increased degradation (Zhuang et al., 2019). However, decreased levels of PDH were only observed in mousebird heart, whereas in skeletal muscle tissue (the tissue that demonstrated by far the largest increase in S232 phosphorylation) PDH levels remained stable during torpor. This suggests that any effect on PDH activity by S232 is likely caused by the phosphorylation itself in mousebirds and not by decreased stability resulting from addition of the phosphate residue. S232 phosphorylation (referred to as phosphorylation site 3 in some literature) is known to specifically inhibit PDH activity by decreasing affinity for the thiamine pyrophosphate cofactor (Korotchkina & Patel, 2001a). Phosphorylation at this site is not only associated with a decrease in PDH activity but has also often been associated with functions not directly related to inactivation of the complex, such as decreasing the functionality of PDH phosphatases that can remove a phosphate group from the S293 site to reactivate the enzyme. Therefore, it is possible that the addition of the phosphate at the S232 site may also be present as a safeguard to prevent unwanted activation of the complex during torpor which would increase metabolic rate (Sale & Randle, 1982). Alternatively, the reduction of PDH phosphatase activity during early torpor could potentially result in an accumulation of phosphorylation on S293 if torpor were allowed to continue for longer periods of time than were tested in these experiments.

Increases in S232 phosphorylation without any other significant phosphorylation changes across a variety of tissues is unusual given that the different PDHK isozymes have different tissue distributions (Klyuyeva et al., 2019). Additionally, the PDHKs can be responsive to common factors, such as increased cellular levels of ATP. The transcription factor HIF-1α is known to activate transcription of PDHK1 and serves as a means of reducing flow of carbohydrates into the Krebs cycle (Kim et al., 2006). Increased protein expression of HIF-1α is known to be a feature in muscle tissue in the 13-lined ground squirrel (*I. tridecemlineatus*) in response to bouts of torpor (Morin & Storey, 2005). While it is not currently known whether a similar response exists in mousebirds, given that PDHK1 is the only known kinase capable of phosphorylating at the S232 site, HIF1-α could be a likely upstream regulator of the changes observed here.

## 5 CONCLUSIONS

The results from the current experiments have demonstrated that torpor in the mousebird is accompanied by a substantial increase in the phosphorylation of the residue homologous to S232 in the PDH E1α subunit. These results are somewhat surprising, given that caloric restriction typically results in increased phosphorylation of all the regulatory serine phosphorylation sites in most species but in the mousebird undergoing torpor only this one site was found to have elevated phosphorylation. This evidence suggests that phosphorylation at this specific site is a likely regulatory mechanism for reducing the catabolism of carbohydrate fuels during torpor in the mitochondria. The present results also demonstrate the significance of PDHK1 in controlling S232 phosphorylation since high levels of S232 phosphorylation proved to be good predictors of PDHK1 cellular abundance between tissues.

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## 7 CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

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