Role of Akt signaling pathway regulation in the speckled mousebird (*Colius striatus*) during torpor displays tissue specific responses

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Highlights

- Avian torpor associated with tissue specific trends in kinase phosphorylation.
- Increase in TSC2 phosphorylation observed in brain, kidney, and liver in torpor.
- General reduction in phosphorylation of multiple targets in Akt-mTOR in muscle.
- Trends suggest decrease in protein translation during torpor in most tissues.

Abstract

Pronounced heterothermic responses are relatively rare among birds. Along with taxa such as hummingbirds and caprimulgids, the order Coliiformes (mousebirds) is known to possess the physiological capacity for torpor. During torpor, body temperature is greatly reduced and a bird becomes unresponsive to external stimuli until external temperatures return to more favorable conditions. Under such conditions, these birds are forced to rely only on their internal fuel storage for energy and show great reduction in metabolic rates by decreasing energy-expensive processes. This study investigated the role of the key insulin-Akt signaling kinase pathway involved in regulating energy metabolism and protein translation in the liver, kidney, heart, skeletal muscle, and brain of the speckled mousebird (*Colius striatus*). The degree of phosphorylation of well-conserved target residues with important regulatory function was examined in both the euthermic control and torpid birds. The results demonstrated marked differences in responses between the tissues with decreases in RPS6 S235/236 phosphorylation in the kidney (0.52 fold of euthermic) and muscle (0.29 fold of euthermic) as well as decreases in GS3K3β S9 in muscle (0.60 fold of euthermic) and GSK3α S21 (0.71 fold of euthermic) phosphorylation in kidney during torpor, suggesting a downregulation of this pathway. Interestingly, the liver demonstrated an increase in RPS6 S235/236 (2.89 fold increase) and P70S6K T412 (1.44 fold increase) phosphorylation in the torpor group suggesting that protein translation is maintained in this tissue. This study demonstrates that avian torpor is a complex phenomenon and alterations in this signaling pathway follow a tissue specific pattern.

Keywords: torpor; signalling kinases; protein translation; initiation factors; avian biology; phosphorylation

1. Introduction

Endothermy is a key physiological feature amongst birds and mammals that has allowed them to colonize a wide variety of habitats by maintaining a relatively constant optimal internal body temperature (Tb) despite external fluctuations in ambient temperature (Ta). While this brings benefits by allowing the animal to remain more active thereby enabling them to colonize habitats that are inhospitable for ectothermic species (Pincheira-Donoso et al., 2013), it also incurs a large metabolic cost since keeping a constant body temperature requires utilizing cellular energy to heat or cool the animal (Bennett and Ruben, 1979). For this reason, many endothermic species may enter periods of metabolic rate depression (MRD) when faced with lowered temperature or caloric restriction. These periods of MRD, depending on their duration, are typically referred to

as either hibernation (extended bouts of days to months) or daily torpor (occurring within the context of the circadian cycle). During these bouts, animals become inactive and their Tb falls to an ambient or otherwise reduced temperature to conserve energy supplies. This process has a wide taxonomical distribution amongst mammals, but appears to be more phylogenetically restricted among birds. To date, eight avian families have been described with at least one member species having the ability to undergo a state of daily torpor (Ruf and Geiser, 2015). With only one known exception, avian MRD involves daily torpor rather than hibernation, and is almost always restricted to the rest-phase of a circadian cycle (Jaeger, 1949; Prinzinger et al., 1992; Schleucher, 2004; Woods et al., 2019).

The mousebirds (order *Coliiformes*) consist of six extant species endemic to Africa that have the ability to undergo torpor. The speckled mousebird (*Colius striatus*) is the most common species in the mesic eastern parts of southern Africa and has been the subject of most studies with regards to torpor in mousebirds. The minimum recorded recoverable Tb of *C. striatus* during torpor is 18.2°C (McKechnie and Lovegrove, 2001). Studies into the physiological responses during torpor have clearly demonstrated that there is a pronounced decrease in metabolic rate at the onset of torpor followed by a gradual decrease in body temperature, showing that the reduction in metabolic rate during bouts of torpor is not merely the result of decreased body temperature (McKechnie and Lovegrove, 2001). Although they possess the physiological capacity for torpor, mousebirds routinely reduce night time energy requirements substantially via communal roosting, with group roosting occurring in a tight cluster (Brown and Foster, 1992; McKechnie and Lovegrove, 2002). Limited evidence suggests that free-ranging mousebirds roosting communally resort to torpor only rarely during periods of inclement weather (McKechnie et al., 2006).

Though the physiological capacity of speckled mousebirds to use torpor has been documented, there remains little research performed on biochemical and molecular mechanisms governing these responses. Signaling kinases and their associated signaling pathways form an integral part of the adaptations responsible for an animal's ability to alter their metabolic rate and which cellular processes are active through phosphorylation of effector proteins (Morrison, 2012; Yan et al., 2016). These kinases are typically grouped into one or more signaling pathways that have downstream targets that effect cellular function. Many different signaling pathways exist and are important in regulating cell growth and division, metabolic rate, response to oxidative stress, apoptosis and numerous other processes. In animals that enter into states of dormancy, overall metabolic rate is greatly decreased to conserve cellular energy reserves and it is likely that multiple pathways are altered in order to achieve this outcome.

The Akt (protein kinase B) signaling pathway plays an important role in regulating various processes including the cell cycle by activating transcription factors that encourage cell proliferation, suppress apoptosis, and promote glucose uptake and consumption (Beg et al., 2017; Chang et al., 2003; Chen et al., 2015). While many signaling pathways are likely regulated in avian torpor, due to its central role in regulating cell proliferation by effecting energetically expensive processes like protein translation, the Akt signaling pathway is ideally positioned to oversee the transition to a torpid state. The activation of Akt is a multi-step process that depends on a cascade of phosphorylation events that occur in response to different stimuli. Insulin serves as an important signal input in activating the Akt pathway. The insulin receptor (IR) is a tyrosine kinase that responds to extracellular insulin at the membrane by self-phoshorylating at tyrosine residues 1162/1163, which allows IR to phosphorylate (activate) insulin receptor substrate 1 (IRS1). Activated IRS1 recruits the p85 subunit of PI-3 kinase to the membrane and thereby

activates the Akt pathway (Luo et al., 2005). This activation is countered by phosphorylation on S312 on IRS1 which inhibits the activity of IRS1(Aguirre et al., 2002). Insulin-like growth factor 1 receptor (IGF1R) is similar in structure and function to IR but is preferentially activated through auto-phosphorylation on tyrosine residues induced by separate peptide hormone, insulin-like growth factor 1 (IGF1) (Jones and Clemmons, 1995). Insulin signaling is thought to be similar in avian species compared to mammals (the taxon most widely studied) in terms of the function of the homologous receptors and kinases, although there is some evidence to suggest that skeletal muscle tissue in displays considerably lower sensitivity to insulin than mammalian muscle (Dupont et al., 2009).

Akt becomes activated by specific serine and threonine phosphorylation sites (notably S473 and T308) and propagates the signal by either directly phosphorylating transcription factors or indirectly by phosphorylating other signaling kinases (Alessi et al., 1997). Activation of Akt typically occurs as a result of phosphorylation on these residues by PDK1 (phosphoinositide-dependent kinase 1) which is recruited to the cellular membrane along with Akt in the presence of phosphatidylinositol-3,4,5-triphosphate (PIP3) (Wick et al., 2000). PIP3 itself is generated by PI3K, a kinase that phosphorylates $PI(3,4)P_2$ (Leevers et al., 1999). Numerous downstream phosphorylation targets of the Akt pathway exist including several transcription factors and enzyme targets. Notably, glycogen synthase kinase 3 alpha and beta (GSK3α and β) are important in regulating carbohydrate usage. The inhibitory phosphorylation on certain residues of GSK3 (S21 of GSK3α and S9 of GSK3β) relieves its inhibitory effect on glycogen synthase (and many other targets), thereby promoting glycogen synthesis (Beurel et al., 2015; Sutherland, 2011) (see Fig. 1). The main role of Akt signaling in regulating protein translation occurs through the phosphorylation of TSC2 at S939 (Inoki et al., 2002). The phosphorylation of TSC2

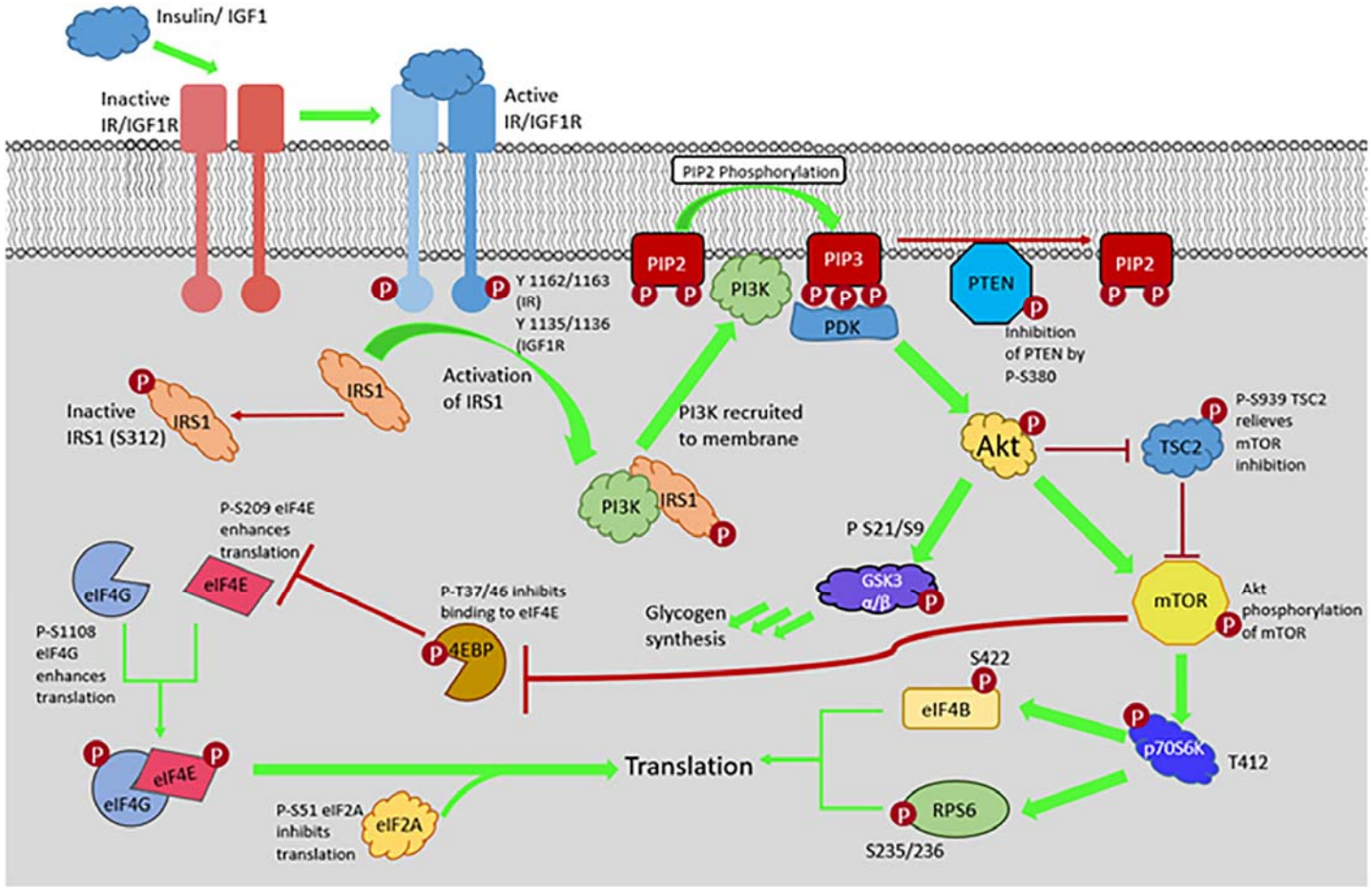

Fig. 1. An overview of the molecular players, their interactions, important regulatory phosphorylation events, and outcomes in the Akt signaling and protein translation pathways. Abbreviations are as follows: IR (insulin receptor), IGF1 (insulin-like growth factor 1), IGF1R (IGFR1 receptor), IRS1 (IR substrate 1), PIP2 (phosphatidyl inositol diphosphate), PIP3 (phosphatidyl inositol triphosphate), PI3K (phosphatidyl-4,5-bisphosphate inositol-3 kinase), PDK (phosphoinositide-dependent kinase), Akt (protein kinase B), PTEN (phosphatase and tensin homolog), GSK3 α/β (glycogen synthase kinase α and β), TSC2 (tuberous sclerosis complex 2), mTOR (mechanistic target of rapamycin), P70S6K (Ribosomal protein S6 kinase beta-1), RPS6 (ribosomal protein S6), eIF (eukaryotic initiation factors), 4EBP (eIF4E binding protein).

relieves the inhibition on mTOR activity, thus allowing for downstream phosphorylation of P70S6K (on T412) and the subsequent phosphorylation of ribosomal protein S6 (RPS6) (on S235/236) and eIF4B that is required to form the pre-initiation complex (Hutchinson et al., 2011; Shahbazian et al., 2006). Countering activation of Akt is the phosphatase PTEN whose primary role is to remove phosphate from PIP3, thereby preventing the recruitment of PDK1 and Akt to the membrane (Chalhoub and Baker, 2009). PTEN activity is known to be inhibited by phosphorylation on S380 is to encourage the activation of the Akt pathway (Tamguney and Stokoe, 2007; Yang et al., 2015). mTOR itself is activated directly by Akt as well as p70S6K through phosphorylation on S2448 (Chiang and Abraham, 2005). Owing to mTOR's position in the signaling kinase cascade and its role in controlling protein synthesis, many cellular, physiological, and even behavioural phenotypes are affected by its regulation depending on the tissue in question. While to date few studies have looked at the regulation of this signaling pathway in bird species, a recent study demonstrated mTOR's role in neurological development in birds and subsequently in song-learning behavior in a passerine species (Ahmadiantehrani and London, 2017).

The phosphorylation of eukaryotic translation initiation factors (eIFs) constitutes an important downstream function of mTOR and other signaling kinases by regulating protein synthesis. These factors are critical for the formation of a pre-initiation complex that aids the assembly of the ribosome and guides mRNA toward translation. Phosphorylation of eIF4E on S209 through non-mTOR pathways may alter its affinity for binding to the 5' methyl cap of mRNA and regulating translation (Scheper and Proud, 2002). Subsequently, eIF4E can bind to eIF4G to recruit the 40S ribosomal subunit to the mRNA for the initiation of protein translation. eIF4G function is enhanced by phosphorylation on S1108 (Bolster et al., 2004; Vary et al.,

2007). Regulating the interaction between eIF4E and eIF4G is the eukaryotic translation initiation factor binding protein 1 (4E-BP1) that is phosphorylated by mTOR on T37/46 to inhibit its binding to eIF4E, that would otherwise prevent the recruitment of the ribosomal machinery (Hara et al., 1997). eIF2a is needed for translation since it enables the GTP-dependent transfer of the first methionine-bearing tRNA to the ribosome and it can be inhibited by various protein kinases phosphorylating on S51 (Sonenberg and Hinnebusch, 2009). Finally, eIF4B is another important protein involved in the regulation of protein translation as it helps to unwind RNA's secondary structure along with eIF4A, thereby providing a binding spot for the ribosome to attach. eIF4B function is enhanced through phosphorylation on S422, downstream of the Akt pathway and it is commonly associated with cell growth and proliferation (Shahbazian et al., 2010).

Given that the Akt/mTOR signaling pathway is central to regulating numerous metabolic and non-metabolic processes, it was of interest to see the effect of torpor on this pathway in mousebirds and study whether this pathway is responsible for regulating protein translation in these animals. We hypothesized that Akt signaling pathway will be regulated in a tissue-dependent manner; leading to a global decrease in protein translation.

2. Materials and Methods

2.1. Animal Treatments

Speckled mousebirds, *Colius striatus*, were captured in Pretoria, South Africa using walk-in traps baited with fruit in September of 2016 (late winter / early spring). Birds were housed in an outdoor aviary with food (a variety of fruits) and water available *ad libitum* at the

University of Pretoria Experimental Farm prior to the start of experimentation. Several weeks prior to the experiments, a passive integrated transponder (PIT) tag (Biomark, Boise, ID, USA) was injected into the abdominal cavity of each mousebird to facilitate measurements of body temperature down to a minimum readable temperature of 20 °C. During the subsequent measurements, Tb of each individual was recorded every ~15 s during measurements using a portable transceiver system (model HPR+, BioMark, Biose ID, USA). PIT tags were calibrated in a water bath at temperatures ranging from 39 to 46 °C against a digital thermocouple reader (model RDXL12SD, Omega, Stamford, CT, USA) with Cu-Cn thermocouples (Physitemp, Clifton, NJ, USA). Each individual was then randomly allocated to either a torpid (experimental) or normothermic (control) treatment (5 birds in each group). Birds in the experimental treatment were moved to a different aviary where food, but not water, was withheld from 09:00 onwards on each day for three consecutive days. In order to confirm the thermoregulatory state of each bird, control and experimental birds were placed individually into 4-L plastic chambers within a darkened temperature-controlled cabinet so that body temperature and metabolic rate could be monitored. Metabolic rates were measured indirectly through oxygen consumption, using the same respirometry setup as described by Lubbe et al. (2018). Birds in the experimental group were exposed to an ambient temperature of 10° C whereas the control birds were maintained at 30°C. Approximately 6 hr after the start of measurements, using body temperature metabolic rate data to verify that birds in the control and experimental groups were torpid (Tb < 20°C and lack of response to external stimuli) and normothermic (body temperature greater than 35 °C and normal activity level), respectively, birds were removed from the chambers and immediately euthanized by cervical dislocation. Tissues were dissected immediately following euthanasia and

frozen by immersion in liquid nitrogen. Tissues were then transported to Carleton University (Ottawa, Canada) where they were stored at -72 °C until use.

2.2. Protein Extract Preparation

Homogenization was carried out using 1× lysis buffer included with the Luminex® kits. Frozen samples were homogenized using a Polytron homogenizer 1:5 w/v in lysis buffer (around 50 mg of tissue per sample) with the following additives included to ensure protein stability and inhibit kinase and phosphatase activity: 1 mM Na_3VO_4, 10 mM NaF, 10 mM β-glycerophosphate, 10 µL/mL of protease inhibitor cocktail (BioShop, # PIC002.1). Samples were then subjected to sonication for 15 seconds to further disrupt cellular membranes and lyse the organelles. Following sonication, samples were left to incubate on ice for 30 minutes with vortex every 10 minutes to ensure protein extraction was efficient before being centrifuged at 14,000 x g for 20 minutes at 4°C. The supernatant containing the soluble proteins was collected and stored at -80 °C until use.

2.3. Multiplex Assay

Luminex® assays were performed for the simultaneous detection of phosphorylated targets in the Akt signaling pathway (EMD Millipore, cat. #48-611MAG) as well as the detection of phosphorylated eukaryotic initiation factors important in regulating translation (EMD Millipore, cat. #48-655MAG) according to the manufacturer's instructions. The Luminex® platform utilizes color-coded magnetic beads bearing antibodies that capture analyte to be measured on their surface. A second biotin-labeled antibody recognizes the analyte and this is subsequently attached to a chimeric streptavidin-phycoerythrin protein. Beads are then read by a two-laser system, the first identifies the color-coded bead and the second quantifies the signal

intensity through phycoerythrin detection. Briefly, the antibody capture beads were sonicated for 10 seconds and then vortexed before being diluted to the working concentration. These beads were incubated overnight at 4°C away from light in a 96 well microplate with constant shaking with assay buffer (blank), or tissue lysates diluted to a pre-determined concentration that was found to yield optimal results. The beads were separated from the lysate by using a Handheld Magnetic Separator Block (#40-285). The wells of the microplate were washed three times with assay buffer as instructed by the manufacturer. The biotin labeled antibodies supplied with the kit were diluted to the working concentration with assay buffer and then added to the wells for 1 hour with shaking at room temperature. The wells were then decanted and washed before the addition of a streptavidin-phycoerythrin solution for 30 minutes. After the incubation, amplification buffer (proprietary components) was added for 15 minutes of shaking at room temperature. Beads were resuspended in assay buffer and the measurements were made using a Lumniex 100® machine with data interpreted through xPonent software. Phosphorylated targets detected by the Akt signaling kit were as follows (with the phosphorylation site in brackets): Akt (S473), GSK3α (S21), GSK3β (S9), IGF1R (Y1135/Y-1136), IR (Y-1162/Y-1163), IRS1 (S312), mTOR (S2448), p70S6K (T412), PTEN (S380), RPS6 (S235/S236), TSC2 (S939). Protein targets detected by the protein translation multiplex assay were as follows: eIF-4G (S1108), eIF-4E (S209), eIF4B (S422), eIF-2a (S51), eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1, total amount), and 4E-BP1 (T-37/46). Optimal protein concentrations were determined for each tissue as determined from the respective dilution curve. All protein concentrations were determined using the Bradford assay (Biorad cat. #5000006)(Bradford, 1976) before the start of the experiments.

2.4. Immunoblotting

Owing to poor detection of two of the targets of the Luminex® assays performed, Western blots were utilized instead to quantify the levels of total mTOR and eIF-4E (S209). Protein extracts were normalized to 10 µg/µL and then mixed 1:1 with SDS loading buffer (100 mM Tris-base, pH 6.8, 4% w:v SDS, 20% v:v glycerol, 0.2% w:v bromophenol blue, 10% v:v 2-mercaptoethanol) and boiled for 5 minutes. To detect mTOR, 50 µg of total protein was loaded on 6% polyacrylamide gels and electrophoresed at 180V until the desired separation was reached. Transfer of the proteins to a polyvinylidene difluoride (PVDF) membrane was performed using a semi-dry Trans-Blot® Turbo™ Transfer System for 12 minutes at a constant current of 1.5 A using transfer buffer (25 mM Tris-base (pH 8.8), 192 mM glycine, and 20% v:v methanol). The gel for the eIF-4E (S209) were electrophoresed on 12% polyacrylamide gels. The transfer for the eIF-4E (S209) blots was performed using the same system but for 5 minutes at 1 A. Primary antibodies used were as follows: rabbit anti-mTOR (7C10) monoclonal antibody (Cell Signaling Technology, #2983), rabbit anti-p-eIF-4E S209 (Cell Signaling Technology #97415). PVDF membranes after transfer were blocked using 1.5% non-fat skim milk powder diluted in TBST for 30 minutes while rocking. Following incubation, membranes were washed 3 times for 5 minutes each time with TBST (20 mM Tris base, 140 mM NaCl, pH 7.6, 0.05% v:v Tween-20). Primary antibodies were diluted 1:1000 in TBST and applied to the membranes overnight with gentle shaking at 4° C. Following incubation, membranes were washed as described previously. Secondary anti-rabbit IgG HRP conjugated antibody was then applied at a concentration of 1:5000 for 30 minutes to each membrane. After washing three more times to removes excessive secondary antibody, the membrane was visualized using enhanced chemiluminescence by mixing 1:1 30% hydrogen peroxide and Luminol solutions. A Chemi-

Genius BioImaging system (Syngene, Frederick, MD, USA) was used to expose the membranes Blots were then stained with a Coomassie brilliant blue solution (0.25% w/v Coomassie Brilliant Blue, 7.5% acetic acid v/v, 50% methanol) to be used as a loading control. Quantification of the band intensity was determined by using Gene Tools 4.3.8.0 software and each protein band was adjusted using manual background correction on a nearby unoccupied area of the blot.

2.5. Statistics

For Luminex® assays and western blotting, the median fluorescence intensity (MFI) intensity was used to determine the relative protein levels in each tissue. For Western blots, the band intensity obtained from the ECL signal was standardized against a group of stably expressed proteins in the same lane on the Coomassie stained membranes. This method of standardization was shown to be more accurate compared to using a single housekeeping gene (Eaton et al., 2013). Data obtained from Luminex® assays and western blotting were normalized to their respective control group for easier comparison. All data represent a mean of 4 independent biological replicates ± SEM from different animals randomly chosen from the 5 control and the 5 torpid animals due to limitations in the amount of reagents available in the assay kits used. Data was determined to be statistically different by Student's t-test when $p < 0.05$. Data was subjected to Bartlett's test of equal variance to determine the appropriate t-test to be used. If the two groups demonstrate unequal variance (i.e. $p < 0.05$ by Bartlett's test) then a heteroscedastic t-test was employed, otherwise a homoscedastic t-test was used. Data and statistical analysis were performed using RBioplot and bar charts were also created using the same software (Zhang and Storey, 2016).

3. Results

Liver demonstrated a few changes in the phosphorylation states of targets in the Akt signaling pathway (Fig. 2A). Notably, there was a prominent increase in the relative amount of RPS6 S235/236 (2.89-fold increase) in the torpor relative to the control. This was accompanied by moderate increases in phosphorylated p70S6K T412 and TSC2 S939 (1.43 and 1.51-fold respectively) levels in torpor relative to the control. Moreover, PTEN T412 levels decreased to 0.81 fold of the control value during torpor. No significant changes were noted in the relative degree of phosphorylation in the initiation factors or 4E-BP1total protein levels in the liver during torpor (Fig. 3A).

In the kidney, several significant changes in the Akt signaling pathway were noticed. Levels of RPS6 S235 phosphorylation and GSK3β S9 phosphorylation decreased 0.52 and 0.86 fold relative to the euthermic control values respectively compared to torpor animals (Fig. 2B). Total protein levels of mTOR were also decreased during torpor relative to the control (0.57 fold of control levels). Phosphorylation of the translation initiation factors was similar between the control and torpid kidneys, except in the case of the initiation factor eIF2A that exhibited a decrease in phosphorylation on S51 to 0.53 fold of the control levels (Fig. 3B).

The heart demonstrated several noticeable changes in response to torpor. Notably, a decrease in IGF1R Y1135/1136 (0.52 fold of the control) and an increase of 1.59-fold in IR Y1162/1163 phosphorylation during torpor were observed (Fig. 2C). A decrease in PTEN S380 phosphorylation to 0.81 fold of the control values was also observed in heart during torpor. Large increases in the phosphorylation status of eIF2A S51 (3.69-fold increase) and eIF4G S1109 (1.47-fold increase) were observed during torpor compared to the control group (Fig. 3C).

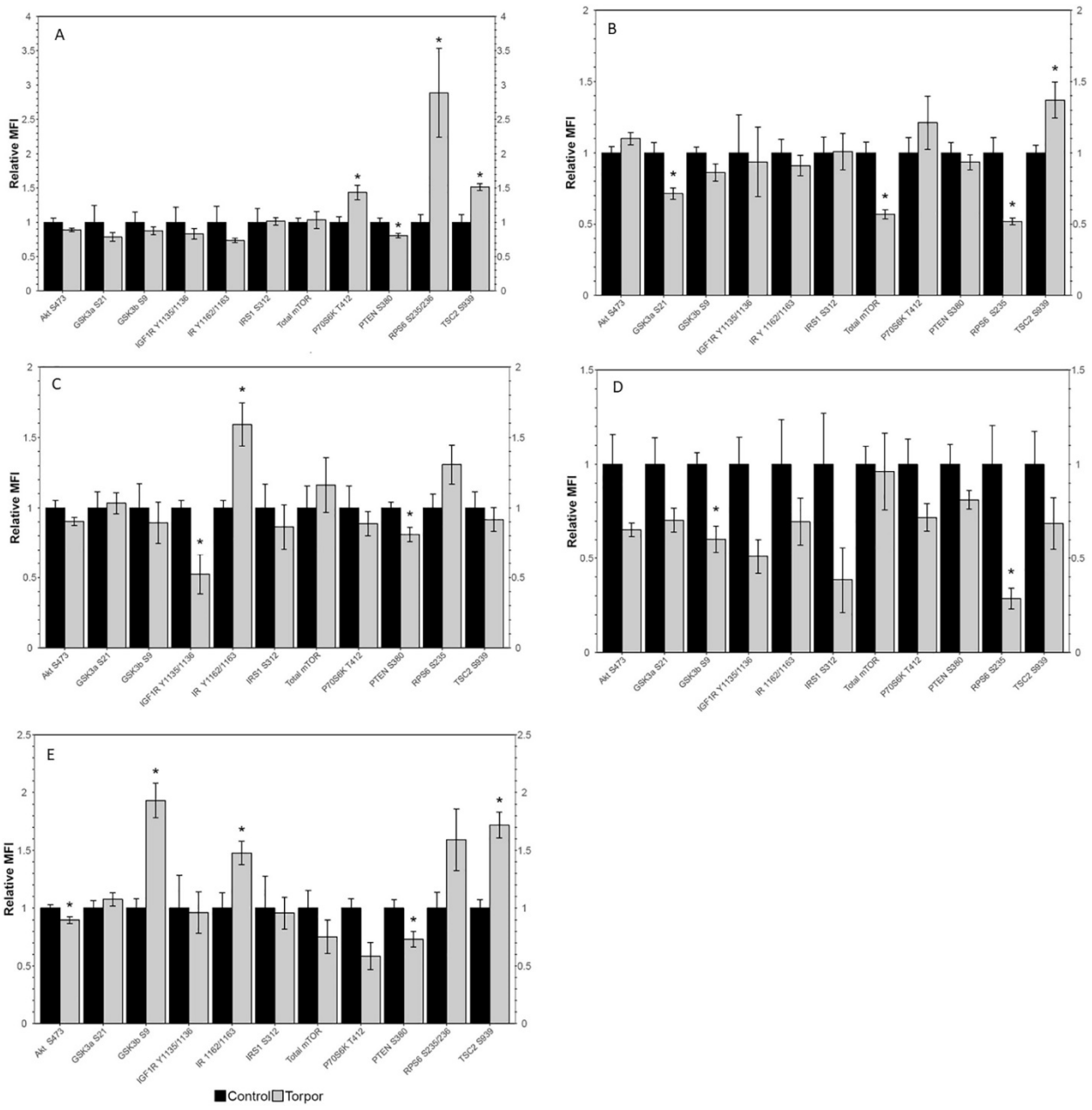

Fig. 2. Relative degree of specific phosphorylation sites of regulatory kinases and targets of the Akt signaling pathway comparing control to torpor states of various tissues from speckled mousebird (*Colius striatus*). The relative protein phosphorylation was normalized to the control sample in each protein target so that the euthermic value was equal to 1. Data represent the mean ± standard error of the mean, $n = 4$ independent tissue samples from separate animals. *Indicates that the torpor value is significantly different from the control ($p < 0.05$, Student's *t*-test). Panels are as follows: A: liver, B: kidney, C: heart, D: muscle, E: brain. Black bars represent the euthermic values for a given parameter and the grey bars the corresponding torpor value.

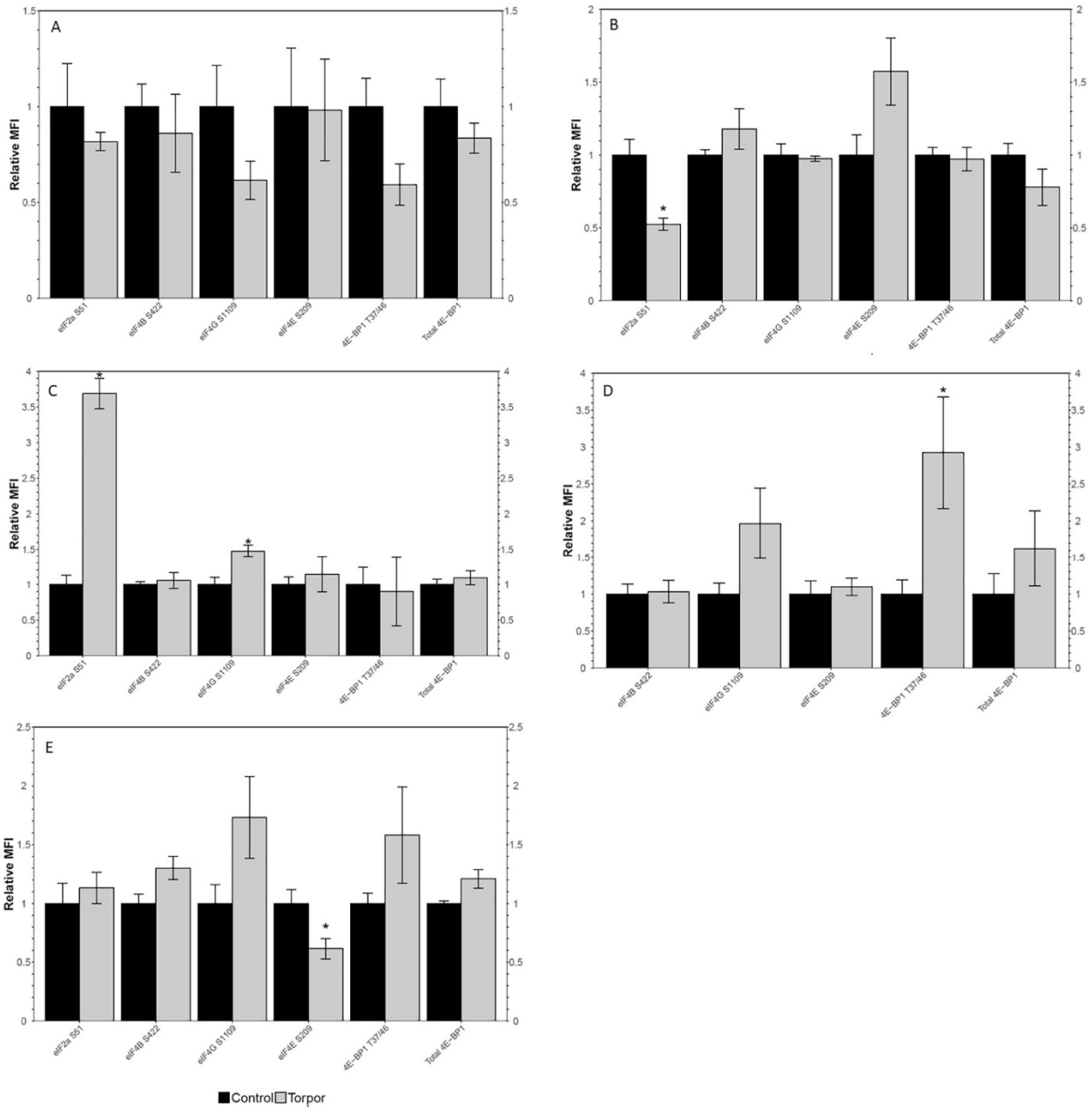

Fig. 3. Relative degree of phosphorylation of specific eukaryotic initiation factors of translation and total protein levels of proteins related to translation in various tissue protein samples from euthermic and torpid speckled mousebirds (*Colius striatus*). The data was normalized against the mean of the control sample for each parameter measured with the mean of the control set to 1. Data are the mean ± standard error of the mean, $n = 4$ independent tissue samples. *Indicates that the torpor value is significantly different from the control ($p < 0.05$ Student's t-test). Panels are as follows: A: liver, B: kidney, C: heart, D: muscle, E: brain. Black bars represent the euthermic values for a given parameter and the grey bars represent the corresponding torpor value.

In the muscle there appears to be a generalized reduction in phosphorylation across numerous targets, although some changes are not significant. A large reduction in the phosphorylation levels of RPS6 S235 (0.29 fold of the control) was noted along with moderate reductions in GSK3β S9 (0.60 fold) during torpor in skeletal muscle (Fig. 2D). Interestingly, an increase in phosphorylated 4E-BP1 (T37/46) level was observed during torpor without a significant increase in the total amount of 4E-BP1 (Fig. 3D).

A 0.9 fold decrease in Akt S473 and a 0.73 fold decrease in the phosphorylation of PTEN S380 was observed during torpor compared to the control group in the brain. This was contrasted with increases in GSK3β S9 (1.93-fold), IR Y1162/1163 (1.48-fold), and TSC2 S939 (1.72) phosphorylation levels in the same tissue (Fig. 2E). A moderate decrease (0.63 fold) was observed in the relative phosphorylation level of eIF-4E S209 during torpor compared to the control group) (Fig. 3E).

4. Discussion

The Akt signaling pathway is known to be regulated differentially in other species undergoing metabolic rate depression in an often tissue specific manner. For example, the strategies employed in daily torpor by the grey mouse lemur involve regulation in fuel energy consumption, notably levels of phospho-insulin receptor substrate (IRS-1) was upregulated in skeletal muscle and phospho-insulin receptor was found at lower levels in white adipose tissue, suggesting reduction in insulin signaling, whereas the opposite trend was observed in the liver (Tessier et al., 2015). Seasonal hibernation in the 13-lined ground squirrel was associated with strong reductions in p-Akt, and p-mTOR in the skeletal muscle, suggesting that this pathway is crucial for metabolic reduction in this species (Wu and Storey, 2012). While seasonal hibernation in a small mammal likely differs greatly from bouts of daily torpor in a mousebird

species due to the physiological differences between the animals themselves and also the adaptions in question (bouts of seasonal hibernation may extend for week at a time and is accompanied by drops to near freezing body temperatures), this study provides evidence to support the notion that this pathway is an important facet of metabolic rate depression. Additional studies have demonstrated significant roles of Akt signaling in numerous organisms capable of undergoing metabolic rate depression including the African clawed frog (*Xenopus laevis*), the wood frog (*Rana sylvatica*), a South American marsupial (*Dromiciops gliroides*), and a species of bat (*Myotis lucifugus*) (Eddy and Storey, 2003; Luu et al., 2018; Wu et al., 2017; Zhang and Storey, 2013). Due to the paucity of avian species capable of entering torpor, to the authors' knowledge, this is the first study investigating Akt pathway regulation in heterothermic responses in an avian model. While multiple differences were observed between the euthermic and experimental group, an important note to make in this study is that there are likely multiple differences between the euthermic and torpid animals that were not statistically different due to the relatively low sample size (n=4 animals) in this study. Therefore, these results should not be interpreted as a comprehensive overview of the differences observed between these two states, but rather as highlights of the most significant changes.

The current study investigated the role of post-translational modifications, specifically phosphorylation, in regulating signal transduction through the Akt signaling pathway in avian torpor. Phosphorylation of the signaling kinases in these pathways is a well-conserved mechanism in vertebrates and in eukaryotic organisms in general (Loewith and Hall, 2011; Saxton and Sabatini, 2017). This allows for tailoring the cellular metabolic processes to meet energetic demands of cells during stress and was hypothesized to play an important role in enabling the mousebirds to enter into the dormant state of torpor. The results demonstrated that

torpor does not rely upon universal pathway responses in all tissues measured, but rather displayed tissue-specific responses to torpor. That is, certain tissues are suggested to be more metabolically repressed during torpor than others, while some are even activated during torpor like the liver and the brain.

In the experiments performed in this study Luminex magnetic bead panels were used to assess the relative levels of phosphorylation on key targets within the Akt-mTOR pathway as well as key players involved in protein translation. While the magnetic bead panel relies on antibodies that were designed to recognize human proteins, the phosphorylation sites in this panel represent well-conserved mechanisms of metabolic regulation in vertebrate cells and the same magnetic bead panel used in this study has been used effectively in another distantly related vertebrate species, the African clawed frog (*Xenopus laevis*)(Wu et al., 2017). The genome of *C. striatus* is not fully annotated, but partial predicted sequences for all of the targets were found in the National Center for Biotechnology Information (NCBI) database except for GSK3a. In all of the partial sequences covering the phosphorylation sites of interest, the residue was conserved between humans and mousebirds, suggesting that Luminex magnetic bead panels would also be able to detect phosphorylation on the homologous proteins in the mousebird samples (See Supplementary material). Additionally, the conservation of these key phosphorylation sites suggests that while little research has directly looked at the role of these pathways in avian biology, they likely play similar roles in regulating metabolism. Indeed, a signal was observed for all of the targets in both panels, except mTOR pS2448 and eIF-4E pS209, demonstrating good cross-reactivity between the antibodies and the mousebird targets. The Luminex magnetic bead panels used relies on a two-antibody system, one to capture the analyte on a magnetic bead and the second to report the presence of phosphorylation through an

attached fluorophore. Despite the S2448 in mTOR and S209 in eIF-4E residues being conserved in the *C. striatus* sequences, no signal was detected and this may be due to the capture antibody recognizing a non-conserved region of the proteins, such as the amino termini of both proteins since the mousebird mTOR sequence has a later start site and the eIF-4E sequence is not well-conserved in that region.

Changes in IGF1R Y1135/1136 phosphorylation and IR Y1162/1163 are suggestive of changing patterns of metabolic fuel usage during torpor in the heart. Interestingly tyrosine phosphorylation was decreased in IGF1R during torpor but was increased in IR (Fig. 2C) in the same tissue. This observation may seem counterintuitive given that both of these receptors have similar structure and both function by activating glycolysis. However, these two receptors are thought to activate the expression of different sets of transcription factors with IGF1R having a more profound role in mediating cellular proliferation while IR more directly encourages metabolic pathways such as glycolysis (Cai et al., 2017). This change in phosphorylation could then represent the need to reprioritize fuel usage during torpor to avoid growth and proliferation in the heart while still maintaining an adequate degree of energy production through glycolysis given the heart needs to continue beating even during torpor albeit at a reduced rate (Schaub and Prinzinger, 1999). Translational initiation factors were also affected in this tissue, namely eIF2A and eIF4G with a noticeable increase in phosphorylation (Fig. 3C). Phosphorylation of eIF2A S51 is associated with a decrease in protein translation as well as maintenance of an anti-apoptotic state, suggesting that translation is reduced during torpor in the heart as well as the importance of cell survival (Muaddi et al., 2010). The increase in eIF4G phosphorylation on S1109 is more difficult to explain as this change is usually associated with improved translational function, however the degree of increase in phosphorylation is much lower when

compared to eIF2A, so it is likely that translational repression dominates. The initiation factor eIF2A is known to be phosphorylated downstream of insulin signaling which could be a possible result of the increased IR activation mentioned earlier (Harris et al., 2006). The decrease in PTEN S380 phosphorylation suggests that PTEN is likely more active in response to bouts of torpor in the heart, which may suggest a lowered degree of Akt signaling pathway although phosphorylation of Akt was not significantly lowered during torpor (Wu and Storey, 2012).

In the liver, there was an observed increase in TSC2 S939 phosphorylation that coincided with an increase in phosphorylation of P70S6K and RPS6 during torpor (Fig. 2A). TSC2 is known to be a tumor suppressor whose activity is inhibited by phosphorylation on S939 by Akt, thereby resulting in the relief of mTOR inhibition. Active mTOR will then cause the phosphorylation of several targets including P70S6K T412 and RPS6 S235/236 (Cai et al., 2006; Inoki et al., 2002), which transduce the signal further to other targets. The decrease in PTEN phosphorylation of S380 again suggests a certain degree of inhibitory relief on the Akt signaling pathway in the liver tissue (Yang et al., 2015). Taken together this may suggest a promotion of protein translation in this tissue during torpor in mousebirds, although phosphorylation of regulatory sites on initiation factors remained constant through torpor. Perhaps, a basal level of translation is necessary in the liver to maintain the production of pro-survival genes. Increases in phosphorylation of TSC2 on S939 have been noted in the past in the liver of marsupial species monito del monte, *Dromiciops gliroides*, during torpor (Luu et al., 2018).

Some similar trends were observed in the brain tissue of mousebirds (Fig. 2E) when compared with the liver (Fig. 4) with increase in TSC2 S939 and a decrease in PTEN S380 phosphorylation in this animal. The brain additionally had increased phosphorylation on IR

Y1162/1163 and GSK3β S9. Similar results were seen in *D. gliroides* with regards to increases in GSK3β phosphorylation on S9 and increased phosphorylation on TSC2 S939 in brain tissue (Luu et al., 2018). GSK3β inhibition has been previously found to help ameliorate brain damage resulting from ischemic strokes in animal models and the increased phosphorylation of this regulatory kinase may be an important player in the brain of animals with reduced metabolic rate (Chuang et al., 2011; Xiao et al., 2017). The role of TSC2 is complicated, however increased phosphorylation on S939 is generally thought to be mediated by Akt and is related to relief of inhibition on mTOR and encourages cell growth and survival (Kang et al., 2011). Regulation of TSC2 is often integrated through energy signaling pathways such as IR, and it is therefore feasible that the increase in IR phosphorylation during torpor in the brain may be related to the increase in TSC2 as well (Bartolomé et al., 2010). The decrease observed in phosphorylated eIF-4E S209 in the brain tissue during torpor seems to run contrary to some of the other changes in the brain, since eIF-4E phosphorylation of S209 is associated with cell proliferation due to its function in promoting recruitment of mRNAs to the ribosome through the 7-methyl-guanosine 5' cap region. However it is known that eIF-4E phosphorylation control may be associated with other signaling pathways besides the Akt pathway, especially the MAPK pathway, and therefore the decrease in phosphorylation on this target may not be reflective of the function of the Akt pathway during torpor (Shveygert et al., 2010). 4E-BP is able to bind and inhibit the activity of eIF-4E when in the dephosphorylated state, although no differences were observed in the brain either in terms of total 4E-BP or in phosphorylation.

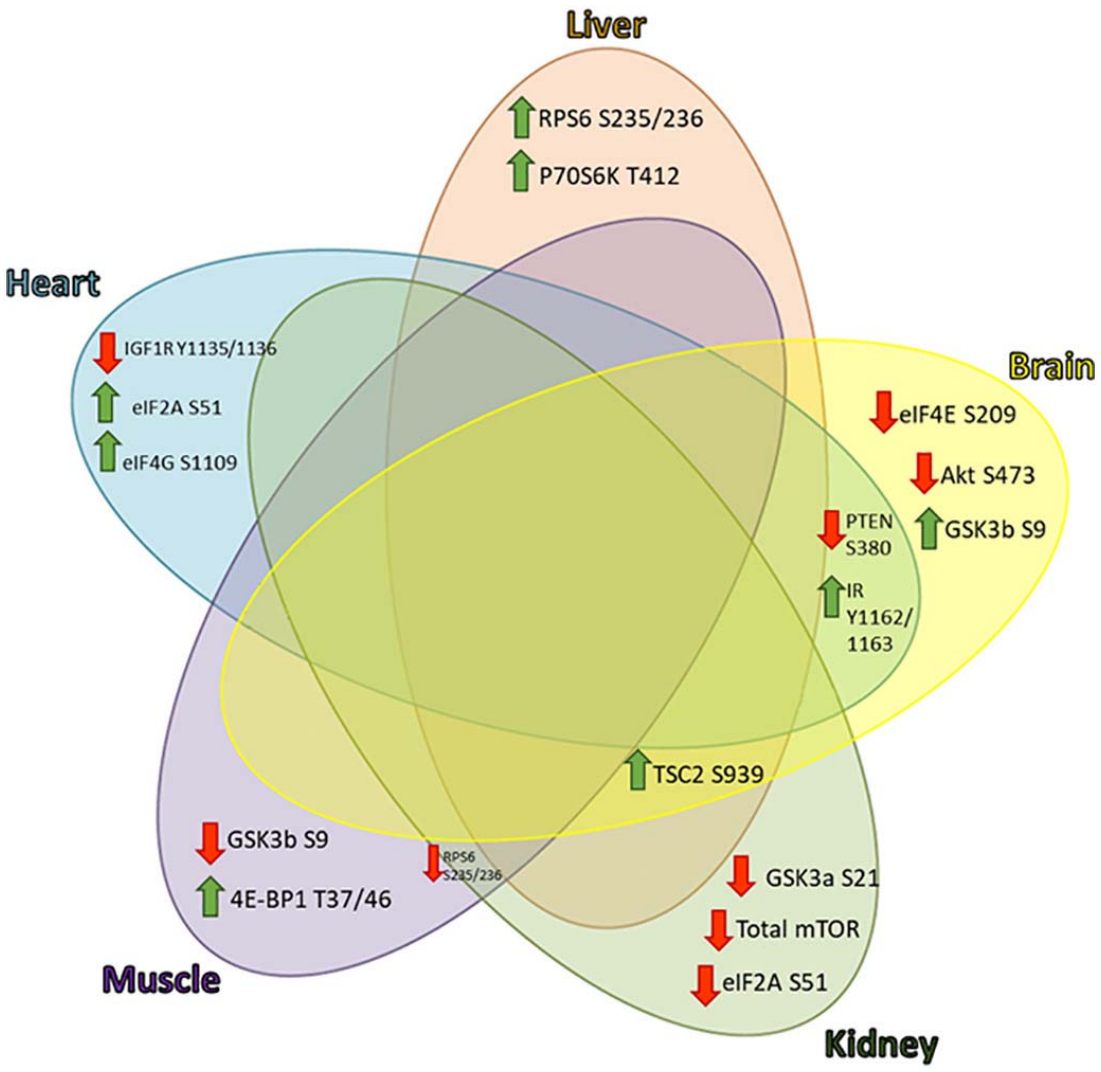

Fig. 4. Visual summary of the differences observed in the Akt signaling pathway and protein translation in the five tissues assayed of *C. striatus*. The trends presented here represent all the statistically significant differences observed in Fig. 2, Fig. 3. Down-arrows represent a decrease in protein phosphorylation on the residue indicated during torpor while up-arrows represent the opposite.

The responses to torpor observed in muscle and kidney differ greatly from those of the brain and liver. In the muscle, a general trend of reduced phosphorylation is noticeable in the targets measured, although this was significant in only a couple of targets. Notably, a decrease in the phosphorylation of RPS6 was observed in both the kidney and skeletal muscle in response to torpor (Fig. 3E). This suggests that these tissues are likely being regulated in a similar manner to

reduce their metabolic rate. The reduction in phosphorylation of the ribosomal protein RPS6 in these tissues would suggest that translation is likely reduced thereby limiting cell growth and proliferation during bouts of torpor, since phosphorylation of RPS6 is known to increase rates of translation (Puighermanal et al., 2017). This observation is contrasted in the kidney where a decrease in phosphorylation of one of the initiation factors, eIF2-α was observed (Fig. 2B). This in theory should promote protein translation by recruiting methionine conjugated tRNAs to the ribosome (Muaddi et al., 2010). Interestingly, eIF2α was shown to have a role in regulating apoptosis in stressed cells. Indeed, studies showed that an increase in eIF2α phosphorylation was associated with promoting a pro-apoptotic response by activating the ATF4-CHOP signaling pathway and decreasing the expression of pro-survival factors such as Bcl-xL (Mounir et al., 2009; Wek et al., 2005). As such, it is possible that a decrease in eIF2α phosphorylation is a cytoprotective response against its role in inducing apoptosis. Alternatively, while the significance of this is unknown when compared to the reduction in phosphorylation of RPS6 in the kidney, a possible explanation could be that mRNAs requiring cap dependent translation could be lowered in expression, while those of non-canonical, cap-independent translational patterns such as internal ribosome entry sites (IRES) could be enhanced. This could possibly allow for a more targeted approach to mRNA translational management during torpor in the kidney by promoting the expression of proteins important for stress survival as has been suggested elsewhere in other animal models (Komar and Hatzoglou, 2011; Nevins et al., 2003). While there was an increase in phosphorylated 4E-BP in muscle, there appears to be a slight increase (although not significant) in the amount of total 4E-BP so any induction of translation would be limited. GSK3β is an important regulatory kinase in these pathways and its reduction in phosphorylation in the muscle tissues is suggestive of an overall reduction in anabolic

processes such as growth and proliferation during torpor. GSK3β plays a critical role, amongst numerous others, in regulating the production of glycogen, with a reduction in phosphorylation on GSK3β a shift in carbohydrate consumption would occur favoring consumption of glycogen in the muscle instead of its production (Oreña et al., 2000). In the kidney, a reduction in the levels of total mTOR were observed during torpor, which may represent a decrease in the signaling of this pathway and could elicit a decrease metabolic expenditure. Reduction in kidney function has been previously observed in a hummingbird species that greatly reduces their metabolic rate during the night in order to reduce dehydration (Bakken, 2004). Alterations in many of the signaling kinases observed therefore are critical in the muscle and kidney by effecting a reduction in metabolic rate allowing for entrance into a dormant state. Additionally, reduction in the degree of GSK3α phosphorylation (S21) was observed in the torpid kidney. GSK3α is known to be phosphorylated by Akt, so decrease in the phosphorylation in the kidney tissue suggests a decrease in the activity of this pathway (Beurel et al., 2015).

5. Conclusions

Torpor is used by many endothermic species to reduce metabolic expenditure through reduction of body temperature and vital processes. The mousebirds represent an interesting taxon to study in this regard as few studies to date have looked at the metabolic underpinnings of avian torpor despite the existence of numerous studies examining the physiological characteristics of this response. The current study demonstrated that management of critical regulatory kinases related to catabolic processes during torpor is governed by means of varying the relative abundance of phosphorylation at highly conserved sites in the Akt signaling pathway. This regulation varies widely between tissues, suggesting that metabolic rate depression does not always constitute a global reduction in metabolic rate but rather takes into account the need to

reprioritize metabolic fuel consumption and the need to activate stress response survival pathways to cope with the immediate cellular insult imposed by torpor and the stresses caused by the return to normal conditions. Taken together these results emphasize the complex nature of torpor in relation to different tissues and also engender additional questions concerning the roles of apoptosis, oxidative stress response, and regulation of cell proliferation during avian torpor.

Declaration of Interest

The authors declare no competing interests.

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