



STRATEGIES OF MOLECULAR ADAPTATION TO CLIMATE CHANGE: THE CHALLENGES FOR AMPHIBIANS AND REPTILES

Kenneth B. Storey and Janet M. Storey
Institute of Biochemistry, Carleton University
1125 Colonel By Drive, Ottawa, ON, Canada K1S 5B6

Tel: (613) 520-3678

Email: kenneth_storey@carleton.ca
jan_storey@carleton.ca

1. Introduction

Over the course of Earth's history, climate has varied widely for numerous reasons, including very recently the actions of humans (Pidwirny, 2006). Through all of this, many organisms adapt and change to the vagaries of climate and at the two "ends of the spectrum" new species arise whereas others go extinct. In this chapter we consider the lives of ectothermic ("cold-blooded") terrestrial vertebrates – the amphibians and reptiles – and how they are affected by and adapt to changing environmental conditions. We will focus particularly on species that have adapted to life in seasonally cold environments and examine some of the specialized biochemical adaptations that support cold and/or freeze tolerance (Storey and Storey, 1992, 2004a; Margasin et al., 2007). We will also survey the range of molecular mechanisms that are available to organisms to make adaptive changes to their biochemistry (and thereby to their viability) in response to environmental stress, with examples from our 30 years of study of the biochemistry of winter cold hardiness of amphibians and reptiles.

All amphibians and the vast majority of reptile species live in terrestrial or freshwater environments, most amphibians being tied to a need for bodies of water or at least damp conditions in their microenvironment. Typically, terrestrial ectotherms have little capacity for migration as a solution when environments become unfavourable (McCallum et al., 2009) and therefore changes in their local environments (temperature, precipitation, pollution, etc.) can put them at risk. They can be particularly sensitive to local thermal conditions that change on daily, seasonal or multi-year time frames and are typically "sandwiched" between upper and lower critical temperatures in determining their own activity/viability (Hillman et al., 2009). Furthermore, amphibians and reptiles are also affected by secondary consequences of temperature change such as altered availability of food, water (changes in precipitation and evaporation rates), shelter/shade, and suitable egg-laying sites, etc. As such, shifting temperature profiles as a result of climate change have already, and will continue to have, a profound impact on the lives of ectothermic vertebrates affecting individuals, local populations, and whole species, and undoubtedly causing extirpation of some species from various geographic locations and complete extinction of others.

2. Climate change and ectothermic vertebrates

Many amphibian species are in trouble around the world showing both declining populations and/or extinctions and this has been the subject of much research over the last 20 years (Stuart et al., 2004; Hayes et al., 2010). Multiple influences have been identified including atmospheric change (temperature, precipitation), habitat destruction, pollution of waters from agricultural and industrial run-offs, invasive species, and pathogens (e.g. chytrid fungus, parasites). In some studies, all other factors can be eliminated leaving atmospheric (climate) change as the apparent cause of continuing declines in amphibians (and some reptiles) (Wake, 2007). Furthermore, warming temperatures interact with other factors (Hayes et al., 2010) such as by enhancing the spread of chytridiomycosis (Bosch et al., 2007). Some of the problems impacting reptiles and amphibians experiencing sudden climate change are outlined below.

2.1 Survival

Reptiles and amphibians are “cold-blooded” animals and, naively, it may be assumed that rising environmental temperatures due to global warming would be a good thing – higher body temperatures (T_b) should facilitate faster growth, higher activity levels, etc. However, it turns out that ectothermic vertebrates are actually highly adept at managing their T_b and frequently maintain near-constant core T_b values via combinations of basking in the sun and cooling in the shade. Physical performance of these animals is generally greatest at core T_b values of 30-35°C and most are heat-stressed above about 40°C; hence, in hot environments, the availability of shade to keep animals from overheating is key to survival (Kearney et al., 2009). Indeed, when the effects of a 3°C increase in environmental temperature were modelled, very serious negative consequences were predicted, especially for tropical reptile species. In particular, food demands to fuel a higher metabolic rate would increase sharply at the same time as the animals would need much more nonforaging time in the shade to keep from overheating (Kearney et al., 2009). Periods of seasonal inactivity (estivation) due to extreme heat would also have to be extended (requiring greater body fuel reserves to be accumulated in the active season) and the timing of reproduction would need to be altered for optimal egg incubation.

In northern regions, warmer temperatures could have the positive effect of allowing northward range extension of various amphibians and reptiles but there are also negatives to consider. Higher T_b values directly increase metabolic rate, thereby increasing both daily food requirements as well as the body reserves needed to last through the winter. Warmer temperatures can go hand-in-hand with reduced precipitation and elevated rates of evaporation creating an overall drier climate not amenable to some species, particularly amphibians that are highly susceptible to water loss across their skin. A reduced thickness of the winter snowpack could also be devastating for amphibian and reptile species that hibernate at or near the soil surface exposing them to temperatures too low to endure. For example, a thick insulating layer of snow can keep soil surface temperatures from falling below -5°C (survivable by freeze tolerant frogs) even when air temperature above the snowpack hovers around -30°C (not survivable) (Storey and Storey, 2004a).

Survival under changing climatic conditions also depends on whether animals can adjust effectively to new conditions. This may be difficult, especially if the pace of climatic temperature change is high. For example, McCallum et al. (2009) applied a climate change model to predict growth responses of three-toed box turtles over the remainder of this century, forecasting that by 2100 less than 20% of hatchlings would grow in their first year and that they would subsequently show reduced growth rates, lower adult size and reduced fecundity as compared with current populations, all conditions that could trigger an extinction vortex. A recent study of tiger snakes,

Notechis scutatus, showed that the animals had problems adjusting to a new temperature regime. Aubret and Shine (2010) raised young snakes in different thermal gradients representing cold (19–22°C), warm (19–26°C) or hot (19–37°C) options. They found that all groups adjusted their basking behavior so that each maintained similar Tb values (24–25°C). However, when cold and hot groups were switched in their second year of life, neither group seemed capable of adjusting their length of basking. Animals in the cold group that were used to basking the longest continued to do so and this resulted in a higher mean Tb (26.5°C) whereas those in the hot group continued to bask for the shortest time resulting in a lower Tb (23.5°C). This showed a significant effect of early life experience on thermoregulatory tactics and a limited plasticity to adjust to year-to-year variation in ambient temperatures.

2.2. Species range

Warming climates can lead to range extension for some species, for example, expansion northward or to higher altitudes. Other species may see their ranges decline due to competition from southern invaders and limitations to their own range expansion, ie. inability to move above the treeline. Alpine populations could easily become fractured, moving to higher and higher elevations until they are isolated and ultimately extinguished. Indeed, a substantial number of the Neotropical amphibian species that have disappeared in recent years are those living in montane zones; animals are “sandwiched” between inhospitable higher elevations and lowland forests where they cannot compete with the resident species (Stuart et al., 2004). However, the opposite can also occur; studies of Columbia spotted frogs (*Rana luteiventris*) in Montana, showed increased survival and breeding probability as the severity of winter decreased suggesting that ectotherms in alpine or boreal habitats that are living near to their thermal ecological limits would benefit from a warming climate as long as suitable habitats remain intact (McCaffery and Maxell, 2010).

2.3 Reproduction

Temperature change associated with global warming may have its most profound effects on breeding. Most amphibians require an aquatic environment for larval development. In temperate regions, warmer temperatures can have positive impacts such as an earlier start to the breeding season and a faster rate of larval growth. Equally, however, there are negative impacts, particularly for species breeding in ephemeral waters. Reduced meltwater (the result of less snow) and higher rates of evaporation at warmer temperatures will decrease the time (hydroperiod) available for larval development. This means that fewer larvae may reach the minimum size needed for metamorphosis before ponds dry out and those that do will be smaller sized when they transform which is associated with reduced terrestrial survival and fecundity (McMenamin and Hadly, 2010).

Most reptiles rely on the sun to incubate their eggs with a minimum number of heat days needed to achieve hatching. For species with large north-south distributions this can allow two or more clutches of eggs to be raised per year in the south whereas the northern range limit is often determined by insufficient heat days to rear a single clutch. For example, this is true of painted turtles at their northern limit in Algonquin Park in Canada, even though this species can deal with another very difficult challenge – survival of whole body freezing by hatchlings during their first winter (Storey et al., 1988). Therefore, at first glance, global warming could allow beneficial northern range extensions for some species but other factors also determine reproductive success. A critical factor for many reptiles is the phenomenon of temperature-dependent sex determination (TSD) during egg development. In turtles, exposure to lower temperatures during the thermosensitive period produces males whereas higher temperatures produce females; the opposite pattern occurs in some lizards. In a third pattern, males are produced at intermediate temperatures and females at both higher and lower temperatures; this occurs in crocodylians as well as some

lizards and turtles (Hulin et al., 2009; Mitchell and Janzen, 2010). As a result, rising global temperatures could easily skew the sex ratio of various species to the point of demographic collapse (Hulin et al., 2009). Indeed, as little as a 1.5°C change in temperature can be the difference between 100% female and 100% male progeny in alligators (Lance, 2009). With a 3-4°C increase in air temperature, Mitchell et al. (2008) predicted that only males would remain in an island population of tuatara (*Sphenodon guntheri*) by about 2050, effectively extirpating the species.

3. Amphibian and reptile cold-hardiness

In terms of its effects on species biodiversity, various studies predict that climate change will have its greatest effect on tropical species for reasons that include the huge number of species in tropical habitats, the often highly specialized lifestyles, and the high environmental temperatures that are already close to the critical thermal maxima. Dynesius and Jansson (2000) state that climatic oscillations select for vagility (dispersal ability and propensity) and generalism and this makes tropical species particularly vulnerable to climate change due to high specialization, low vagility and small geographic ranges despite the high species diversity of the tropics. In temperate and polar zones there are fewer species overall but individual species can range over vast areas. For example, the wood frog (*Rana sylvatica*) is found across the entire boreal forest of North America and is the most northerly distributed amphibian or reptile with a range far above the Arctic Circle (Figure 1). Wood frogs expanded northwards following the retreat of the glaciers after the last ice age and phylogenetic analyses by Lee-Yaw et al. (2008) show that colonization of the north derived from a limited number of eastern and western refugia with high-latitude refugia in the Appalachian highlands and modern-day Wisconsin having the biggest impact on northern populations. Most amphibian and reptile species in the northern USA and Canada show similar patterns of recolonization from southern refugia (Lee-Yaw et al., 2008). Therefore, as a result of founder effects, colonizers should show high levels of genetic homogeneity in the north (Hewitt 1996) and this could make them particularly susceptible to climate change. Impacts of global warming on northern species could occur in at least three ways: (1) direct effects on individual cold-hardy species, (2) effects on the food species and habitat (e.g. breeding or wintering sites) of cold-hardy species, and (3) competition from less hardy species moving northward as the climate gets milder. In the rest of this article we will focus on the first of these, beginning with a survey the strategies used by amphibians and reptiles for survival in cold climates and then examining the biochemical options that organisms have for adapting to new environmental challenges.

[place Figure 1 about here]

Figure 1. (A) The wood frog, *Rana sylvatica* (aka *Lithobates sylvaticus*), in unfrozen and frozen states. (B) Wood frog distribution in North America, retrieved from <http://www.iucnredlist.org/apps/redlist/details/58728/0>. (C) Profiles of cryoprotectant glucose accumulation in blood and organs of wood frogs during freezing and glucose clearance after thawing. Data in (c) are replotted from Storey and Storey (1986).

Winter presents multiple challenges to amphibians and reptiles. Cold temperatures greatly limit activity and food availability and when environmental temperatures fall below 0°C, animals are at risk of lethal freezing. Most northern and alpine ectotherms choose to hide for the winter in

thermally buffered sites. Some species go underground below the frostline, digging by themselves (e.g. toads) or exploiting natural tunnels or caves (e.g. salamanders, snakes). In the interlake region of Manitoba, for example, garter snakes migrate several kilometers to mass by the thousands in underground caverns in the limestone bedrock (Gregory and Stewart, 1975). These species do not appear to need highly specialized adaptations to endure winter cold but they must be well-attuned to both photoperiod and thermoperiod cues to trigger their autumn movement to hibernacula and their emergence in the spring. Their key adaptation for winter survival may be the accumulation of sufficient body fuel reserves during summer/autumn feeding; enough is needed to fuel not only a 6-9 month period of cold inactivity but also an intense period of spring breeding that frequently occurs before eating resumes.

Other ectothermic vertebrates hibernate underwater. By doing so, various frogs, turtles and snakes place themselves advantageously in a near-constant 0-4°C environment although they are still vulnerable to winter-kill if the water freezes to the bottom. The main challenge of this winter strategy is oxygen availability for two reasons: (a) these are lung-breathing animals, and (b) organismal respiration (microbes, plants, animals) depletes oxygen in ice-locked bodies of water leading to hypoxia or even anoxia. The problem of acquiring oxygen is solved for submerged frogs by simply switching from lung breathing to oxygen uptake across their skin (Tattersall and Ultsch, 2008). Some turtles also do this and others can absorb oxygen across other surfaces (cloaca, cloacal bursae, buccopharyngeal) (Jackson and Ultsch, 2010). Because oxygen content is highest in cold water and animal metabolism is lowest, these nonpulmonary solutions to oxygen uptake can be sufficient to sustain aerobic metabolism over the winter.

Other turtles have optimized anoxia tolerance to deal with long term submergence. For example, painted turtles (*Chrysemys picta*) can survive in deoxygenated cold water for ~3 months (Jackson and Ultsch, 2010) and, indeed, these and red-eared sliders (*Trachemys scripta elegans*) are widely used in biomedical studies to discover the molecular adaptations that allow survival of long-term oxygen deprivation by vertebrate brain and heart (Lutz and Milton, 2004; Storey, 2007; Bickler and Buck, 2007). The adaptations involved include acquisition of high levels of fermentable fuels in organs (massive stores of glycogen in liver), use of the shell as a site to store and buffer the products (lactate, H⁺) of anaerobic glycolysis, and strong metabolic rate depression (Storey, 2007; Jackson and Ultsch, 2010). Indeed, the metabolic rate of a turtle submerged in cold deoxygenated water is only about 10% of the value in air at the same temperature (Herbert and Jackson, 1985) and just 0.6% of the metabolic rate in air at a common summer temperature of 20°C (Jackson and Ultsch, 2010). Metabolic rate depression is not just a feature of anoxia tolerance but is a major contributor to energy savings in many species confronted with environmental stress including amphibians and reptiles estivating in hot climates (Storey, 2002; Storey and Storey, 2010) and those frozen solid over the winter months (discussed below).

The third strategy for winter survival by terrestrial ectotherms is to endure subzero temperatures, by one of two ways (Storey and Storey, 1988). The first is to supercool, using strategies to maintain body fluids in a liquid state when T_b falls below its equilibrium freezing point (FP). The second is freeze tolerance, using strategies to manage and endure the conversion of as much as 50-70% of total body water into extracellular ice. Freeze tolerant animals endure the penetration of ice into all extracellular spaces (e.g. abdominal cavity, bladder, brain ventricles, eye lens, plasma) and survive the interruption of all vital functions including breathing, circulation, and nerve and muscle activity, regaining these in a coordinated manner upon thawing. The equilibrium FP of the body fluids of terrestrial ectotherms is typically only about -0.5°C. Frogs can rarely supercool below -2°C because their highly water-permeable skin allows easy nucleation of body water due to contact with environmental ice or the action of ice-nucleating bacteria on skin or in gut. Hence, several species of frogs that hibernate on the forest floor have developed freeze

tolerance. The best studied is the wood frog, *R. sylvatica* (Figure 1), but other North America species that are freeze tolerant include gray tree frogs, *Hyla versicolor* and *H. chrysoscelis*, spring peepers *Pseudacris crucifer* and chorus frogs, *P. triseriata* (Storey and Storey, 1992). Two freeze tolerant amphibians are also known from Eurasia: moor frogs, *Rana arvalis* (Voituron et al., 2009) and Siberian salamanders, *Salamandrella keyserlingi* (Berman et al., 1984). Among reptiles, ecologically-relevant freeze tolerance has been reported for the European common lizard, *Lacerta vivipara* (Voituron et al., 2002) and for several turtle species. Both oviparous and viviparous strains of *L. vivipara* occur; they have equivalent supercooling capacities but only the viviparous strain can endure prolonged freezing. This correlates well with the more northerly distribution of the viviparous form (up to 69°N), compared with 47°N for the oviparous form, and supports the theory that cold climatic conditions are a key selective force favouring viviparity in reptiles (Voituron et al., 2004). Box turtles (*Terrapene carolina*, *T. ornata*), at ~500 g in mass, are both the largest known freeze-tolerant animals and the only adult turtles that display this adaptation (Storey and Storey, 1992). However, hatchlings of several northern turtle species that spend their first winter on land are freeze tolerant including painted turtles (*Chrysemys picta*), Blanding's turtles (*Emydoidea blandingii*), diamondback terrapins (*Malaclemys terrapin*), and ornate box turtles (*T. ornata*) (Storey and Storey, 1992; Costanzo et al., 2008). Hatchlings of some other species rely instead on a capacity to supercool and resist ice nucleation; the map turtle, *Graptemys geographica*, is a good example (Baker et al., 2003). Interestingly, both cold hardiness strategies have been reported for painted turtle populations from different geographic locales; some populations of *C. picta* show well-developed freeze tolerance whereas others show extensive supercooling (to -10°C or lower) (Storey, 2006; Costanzo et al., 2008).

Freeze-tolerant vertebrates tend to be those that spend the winter on land at or near the soil surface. As compared with aquatic hibernation, the benefits of terrestrial hibernation can include predator avoidance over the winter months (important for neonatal turtles) (Gibbons and Nelson, 1978), the ability to start breeding very early in the spring, and possibly also energy savings due to an overall lower metabolic rate than is possible in aquatic sites. However, the low temperature limit for most freeze-tolerant vertebrates is only about -6°C (and often higher) so survival also depends on substantial insulation from layers of organic litter and snow to defend against air temperatures above the snowpack that may fall to much lower values. These species may be particularly vulnerable to global warming because milder winters will reduce the snowpack, lowering its insulation value and likely exposing animals to lethal subzero temperatures. A reduced snowpack also means reduced meltwater in the spring to form the ephemeral ponds used for breeding by terrestrially-hibernating frogs as well as reduced time available for tadpole development before ponds dry out. This might be offset by the effects of higher temperature on the rate of larval development but optimal development of cold-hardy species might actually be geared to a cool temperature range.

The development of freeze tolerance was likely aided by pre-existing physiological and biochemical capacities that are broadly present in reptiles and amphibians. The hypoxia/anoxia tolerance displayed by many species would aid ischaemia endurance caused by plasma freezing and the general tolerance of amphibians for wide variation in body water content would help them endure the cell dehydration and volume reduction that occurs when water exits to join extracellular ice crystals. Metabolic adaptations that generally promote anoxia or dehydration tolerance would have been brought into play during the development of freeze tolerance. For example, the accumulation of high concentrations of urea in blood and tissues is a well-known colligative defense against water loss in estivating anurans (Storey, 2002) and also occurs in freeze tolerant frogs (Costanzo and Lee, 2005). Hence, it is not surprising that rudimentary freezing survival has been reported in a number of species (other than those above) if the freeze duration is short (several

hours maximum), temperatures are mild (e.g. -1°C to -2°C), ice accumulation is low (usually $<20\%$ of total body water), and ice is restricted to peripheral tissues (skin and skeletal muscles) (Storey and Storey, 1992). Such short term tolerance may be useful for animals experiencing an unexpected overnight frost but specific biochemical adaptations are needed to push freezing survival to days or weeks, the ecologically relevant requirement to survive through the winter.

4. Principles of biochemical adaptation

Adaptation allows organisms to, as much as possible, sustain homeostasis, preserving the underlying web of biochemical reactions that constitutes life but, when necessary, make fundamental adjustments to selected systems to cope with new realities. All animal adaptation can ultimately be traced to the molecular level involving mechanisms such as (a) changes to gene sequences that dictate the structures and properties of proteins, (b) the evolution of new gene products that allow quantum leaps to be made in species capability (e.g. development of antifreeze proteins), (c) changes to regulatory mechanisms that adjust type, amount, timing and signal responsiveness of gene expression, protein translation, and protein/enzyme function, and (d) changes to multiple other features of the cellular environment within which the “business” of life takes place (e.g. levels of low molecular weight metabolites and ions, modification of buffering capacity, membrane lipid composition). Our exploration of the biochemical adaptations that underlie the acquisition of cold hardiness and freeze tolerance in reptiles and amphibians illustrates the types of molecular tools and mechanisms that animals can bring to bear in adjusting to environmental stress. The remainder of this chapter will focus on this repertoire of molecular mechanisms.

5. Enzymatic and metabolic adaptation

5.1 Low molecular weight protectants

A fundamental principle of survival in almost all freeze tolerant vertebrates and invertebrates (except reptile species) is the synthesis of high concentrations of low molecular weight cryoprotectants that act colligatively to limit the loss of water from cells and also protect/stabilize macromolecules. Freeze tolerant frogs typically use glucose or glycerol whereas cold-hardy insects rely on polyhydric alcohols (glycerol, sorbitol, etc.) (Storey and Storey, 1988). For example, Figure 1C shows the high level of glucose accumulated in wood frog organs during freezing; glucose synthesis from liver glycogen is triggered within 5 min of ice nucleation on the skin. The synthesis of high concentrations of small protectants (sugars, polyols, amino acids, urea, etc.) is a widespread animal adaptation to water/osmotic stress in response to many stresses (dehydration, hypersalinity, freezing, etc) (Yancey, 2005) and is a defense strategy that would be quite simple to implement on an evolutionary scale because it typically involves common metabolites and regulatory controls that are implemented by quantitative rather than qualitative changes. For example, freeze intolerant leopard frogs (*Rana pipiens*) that hibernate underwater show a hyperglycemic response to dehydration (a 24-fold increase in glucose in liver); this could be modified quantitatively in freeze tolerant species to produce the increases of up to ~ 300 -fold that are seen in *R. sylvatica* liver during extracellular freezing (Storey, 1997). Indeed, we have shown that the cryoprotectant synthesis response to freezing by wood frog liver is stimulated just as strongly by dehydrating the animals at 5°C .

5.2 Fuels and end products

Quantitative and qualitative changes to body stores of fuel reserves is a feature that should also be rapidly adaptable in response to environmental stress or climate change. Anoxia tolerant and cold-hardy animals accumulate higher reserves of glycogen than do intolerant species to meet their need for carbohydrate fuels whereas estivating species and mammalian hibernators store massive fat reserves to support aerobic metabolism over many months of dormancy. If climate change alters the percentage of the year that animals can be active and foraging, then adjustments will need to be made to ensure that sufficient body fuel reserves are accumulated (or food is cached) to sustain viability over the nonactive season. The end products of energy metabolism are also a concern, especially if species are forced to use anaerobic metabolism for extended times. Again, pre-existing solutions can be modified quantitatively. This seems to be the strategy of anoxia tolerant turtles that winter underwater. All turtles can accumulate lactate during prolonged diving but species like *C. picta* or *T. scripta* that winter underwater for months have taken this to the extreme with two main modifications: (a) massive amounts of lactate are moved into shell and bone for storage, and (b) calcium carbonate is dissolved from shell and bone to buffer the acid load associated with lactic acid production (Warren and Jackson, 2007).

5.3 Enzyme and protein regulation

Regulation of enzyme activity and the actions of functional proteins is one of the most important levels at which adaptive change can be accomplished (Hochachka and Somero, 1984; Storey, 2004a). This can take place on many levels. Coarse controls on the amounts of enzymes/proteins or on the types of isoforms expressed are achieved by altering gene expression (see below) or protein degradation. Fine controls are achieved by multiple methods including changes in (a) levels of substrates, products and allosteric effectors, (b) posttranslational modification of proteins, (c) protein-protein binding interactions, (d) subcellular localization, and (e) enhanced stability or longevity of proteins via the action of protein chaperones and low molecular weight stabilizers (Storey, 2004a). Posttranslational modifications of enzymes are particularly useful for making stable changes to the activities and properties of enzymes and functional proteins in response to environmental stress and would certainly be involved (at least as an early response) in animal mechanisms of dealing with climate change. Multiple forms of covalent modification exist that can make stable modifications to enzyme function in response to signals and stresses; these include phosphorylation, acetylation, methylation, ubiquitinylation, SUMOylation, GlcNAcylation and many others.

As a mechanism of stress-responsive differential regulation of enzymes, reversible protein phosphorylation (RPP) via the actions of protein kinases and protein phosphatases, is by far the best-known. RPP is a central mechanism by which animals adjust the activities of enzymes and functional proteins in response to environmental stresses including cold, freezing, oxygen limitation and desiccation (Storey 2004a). Perhaps the earliest identification of RPP in a stress response was as the mechanism of cold-activation of glycogen phosphorylase to trigger cryoprotectant synthesis in insects (Ziegler et al., 1979) and later in wood frogs (Storey and Storey, 1988). A huge number of cellular proteins are targets of RPP and the mechanism is now known to be widely used for coordinating the action of multiple cell functions in response to signals and stresses. Indeed, RPP is the primary mechanism that accomplishes metabolic rate depression, reprioritizing and turning down/off energy expensive cell functions (e.g. ATP-driven ion pumps, transcription, translation) under stress conditions as well as rationing fuel supplies (Storey and Storey, 1990, 2007).

A recent study of creatine kinase (CK) from skeletal muscle of wood frogs provides an instructive example of the methodologies that can be used to determine whether an enzyme

undergoes stress-responsive RPP and to discover the consequences phosphorylation for enzyme function (Dieni and Storey, 2009). CK catalyzes a reversible reaction that controls phosphagen pools and their interconversion with ATP ($\text{creatine} + \text{ATP} \leftrightarrow \text{creatine-P} + \text{ADP}$) and is particularly important in muscle where creatine-P hydrolysis instantly supplements ATP levels when contraction is initiated. Evidence for covalent modification is usually derived first from identification of one or more stable significant changes in the properties of an enzyme between control and stressed states. In this case, CK isolated from muscle of frogs frozen for 24 h at -3°C showed significant differences in its maximal activity (35% higher) and affinity (K_m) for creatine (29% lower) compared with CK from 5°C acclimated control frogs (Figure 2b,c). An increase in activity could derive from a greater amount of CK protein but immunoblotting found no change in total CK protein between control and frozen states. Strong evidence of probable RPP can then be derived from monitoring elution profiles of an enzyme off an ion exchange column because phosphorylation alters the net charge on a protein and thereby shifts its elution profile. Fig. 1A shows that frog muscle CK eluted in two peaks from DEAE Sephadex and the percentage of activity in each peak changed in response to freezing; in control muscle 60% of CK was in peak II compared with only 35% in frozen animals. Even stronger confirmation of RPP as the mechanism of stress-responsive stable modification of the enzyme can come from the use of *in vitro* incubations that promote the action of protein kinases versus protein phosphatases. Incubation of control CK under conditions that stimulated protein kinases led to an increase in CK activity (Figure 2B) and a decrease in K_m creatine (Figure 2c) comparable to the changes in properties of CK from frozen muscle. By contrast, stimulation of phosphatase activities in extracts from frozen muscle reduced CK maximal activity and increased K_m creatine to values that mimicked the control situation. Put together the data show that phosphorylation increases CK activity and substrate affinity, that freezing stimulates increased CK phosphorylation, and implicates peak I as the phosphorylated enzyme form (Figure 2A). Further confirmation of stress-responsive changes in phosphorylation state can come from two procedures: (a) incubations under kinase-stimulating conditions in the presence of ^{32}P -ATP followed by immunoprecipitation of radiolabeled enzyme (Dieni and Storey, 2009) or (b) the use of ProQ Diamond phosphoprotein stain to detect the relative amounts of phosphate bound to the two enzyme forms (Bell and Storey, 2010). Incubation studies can also be used to identify the probable protein kinase(s) and phosphatase(s) that act on an enzyme *in vivo* by adjusting the specific components of the *in vitro* incubation mixture. From such studies, we implicated AMP-activated protein kinase (AMPK), calcium-calmodulin dependent protein kinase (CaMK) and protein phosphatases 2B and 2C in the control of frog muscle CK (Figure 2b). Interestingly, other studies found that freezing triggered a 4.5-fold increase in AMPK activity in wood frog muscle (Figure 2d) (Rider et al., 2006) suggesting that this could be the protein kinase that is involved *in vivo* in CK control. AMPK has an important role as the “energy sensor” of cells (responding to rising AMP whenever ATP is depleted) and so it makes sense that it would also regulate CK that buffers ATP levels by mobilizing phosphagen stores.

[place Figure 2 about here]

Figure 2. Regulation of skeletal muscle creatine kinase (CK) from freeze tolerant wood frogs by reversible protein phosphorylation. (A) Elution profiles of muscle CK from control (5°C acclimated) versus frozen (24 h at -3°C) frogs on DEAE Sephadex. (B) Effect of *in vitro* incubations promoting protein kinase (total kinases, AMPK, CaMK) or protein phosphatase (total phosphatases, PP2B, PP2C) action on CK maximal activity as compared with the situation in STOP buffer where inhibitors of both kinases and phosphatases are present to preserve the phosphorylation state in the tissue. (C) Effect of *in vitro* incubations promoting protein kinases or phosphatases on the K_m

creatine of muscle CK. (D) AMPK activity in skeletal muscle of frozen frogs. ^a Significantly different from the corresponding control (or ^b frozen) condition in STOP buffer (no additions), $P < 0.05$. Compiled from Dieni and Storey (2009) and Rider et al. (2006).

5.4 Changes in protein types and levels

Stress-induced changes in the types and amounts of proteins in cells and tissues typically derive from changes in gene expression although other factors are involved (Storey and Storey 2004b). In recent years the methods available to search for gene expression responses to environmental stress have expanded greatly and include multiple forms of DNA array screening (both homologous and heterologous), protein screening by 2D gel electrophoresis and mass spectrometry, and screening methods targeted at subsets of proteins (e.g. transcription factors) (Eddy and Storey, 2008; Storey and Storey, 2010). These are being used effectively in many different ways to highlight the changes in gene and protein expression patterns that are triggered by environmental stress. For example, we have used these approaches to identify genes that are freeze responsive in wood frogs, and freeze/anoxia responsive in hatchling painted turtles (Storey, 2004b, 2006).

Multiple options are open for achieving adaptive responses by proteins to stresses including climate change. Again, quantitative change may be the easiest to initiate and all that is needed in some cases. For example, the amount of glycogen phosphorylase is 12-fold higher in hepatocytes of wood frogs compared with leopard frogs, reflecting the need for rapid cryoprotectant glucose synthesis by the freeze tolerant species (Mommensen and Storey, 1992). Enzymes involved in urea synthesis are also quickly up-regulated when anurans face desiccating or hypersaline conditions (Balinsky, 1981).

Generalized protein responses to stress are also used to preserve/protect existing cellular macromolecules by synthesizing specialized protective agents, both metabolites (discussed earlier) and proteins. The best known of the latter are the so-called heat-shock proteins (HSPs) that act as molecular chaperones to refold proteins that are unfolded or misfolded under stress conditions. Although ubiquitous responses to heat stress in animals, they also respond to other stresses including hypoxia, osmotic stress, UV irradiation, heavy metals, etc. For example, anoxia exposure triggered coordinated increases (1.8-2.9 fold) in the levels of Hsp25, Hsp40, Hsp70, Hsp90 and Hsc70 (the constitutive chaperone) in skeletal muscle of turtles (*T. s. elegans*) (Krivoruchko and Storey, 2010a). Antioxidant defenses are another protective strategy for dealing with environmental stress. Their importance is emphasized in two ways. Firstly, stress-tolerant species typically have higher constitutive activities of antioxidant enzymes than do intolerant species; e.g. activities of most antioxidant enzymes in liver of freeze tolerant wood frogs are 2-3 fold higher than in leopard frogs (and are even higher in liver of anoxia tolerant *T. s. elegans*) (Hermes-Lima et al., 2001). Secondly, tolerant species can show rapid stress-mediated enhancement of antioxidant enzyme expression that typically occurs under the stress condition (while anoxic, frozen, etc) to prepare animals for a burst of reactive oxygen species formation that accompanies the reintroduction of oxygen when animals exit the stress situation (Hermes-Lima et al., 2001). Indeed, appropriate responses by these and other defensive mechanisms form a generalized cellular stress proteome that is found across phylogeny (Kultz, 2005) and gives all organisms basal mechanisms to help preserve viability in the face of climate change or other environmental stresses.

A final mode of protein adaptation that is available to animals to deal with persistent stress is the elaboration of completely new protein types. Because significant evolutionary time is needed to create completely new protein types, this is not a strategy for dealing with rapid climate change

but is a longer term adjustment that can re-optimize species in an altered environment. For example, wood frogs show strong freeze-stimulated expression of three novel genes/proteins that are not found in other animals, even closely related frogs, although their functions are not yet known (Storey, 2004b). The antifreeze proteins (AFPs) of marine fish are excellent examples of some of the options for creating new protein types (Fletcher et al., 2001). In evolutionary terms AFPs are quite young, the need for them having arisen only 10-14 million years when sea level glaciation recurred. Several types evolved rapidly each with a different story. For example, Type II AFPs have been shown to be derived from the carbohydrate recognition domains of C-type lectins, proteins that play a role in immunity by recognizing surface carbohydrates on pathogens. By contrast, antifreeze glycoproteins in Antarctic fish are composed of a repeating glycotriptide that turned out to be derived from a gene segment crossing the first exon-intron boundary of the trypsinogen gene. Trypsinogen is a protein that is normally secreted into the gut lumen which is one of the main sites where antifreeze action is needed because polar fish often consume ice crystals along with their food. Probably the antifreeze protein evolved by exploiting a minor pre-existing antifreeze action of the ancestral protein. The lesson here is that environmental stress can place strong pressures on species to quickly come up with solutions that allow them either re-adjust and preserve life when their environment changes.

6. Regulation of gene expression

Genes define the total biological repertoire that any organism can bring to bear in its effort to survive and adapt to environmental stress. A variety of mechanisms contribute to the regulation of gene expression but we will focus here on selected categories that illustrate important concepts and mechanisms that are applicable to studying gene expression responses to environmental stress including during climate change.

6.1 Epigenetic regulation of DNA transcription

Epigenetics is the study of heritable changes made via mechanisms other than changes in the underlying DNA sequence of genes. Primary mechanisms include posttranslational modifications of histones (e.g. methylation, acetylation, phosphorylation and others) and methylation of cytosine residues in DNA itself (Zhang 2008). Epigenetic mechanisms are best known in the regulation of cell differentiation during development and mostly affect somatic cells over the course of an individual's lifetime. For example, these are the reason why a differentiated liver cell only produces more liver cells when it divides despite having the full DNA complement needed to make any cell type. However, epigenetic modifications that occur in sperm or egg can potentially be carried forward to the next generation providing a means for environmental stresses that alter the DNA or chromatin structure of the parent generation to influence their offspring. Importantly, epigenetic variation may be generated at a much higher rate than equivalent variation in DNA nucleotide sequences, especially under rapidly changing environmental conditions when organisms are under pressure to produce alternative phenotypes (Angers et al. 2010). Accumulating evidence indicates that epigenetic modifications of gene expression could be a significant and exciting new factor in adaptation and evolution (Turner 2009); indeed, over 100 cases of inherited epigenetic variations in bacteria, protists, fungi, plants, and animals are now known (Jablonka and Raz, 2009). A new study links epigenetic modification with climate change. Paun et al. (2010) report that three species of allotetraploid orchids that arose during or since the last glacial maximum show species-specific epigenetic patterns have had a direct impact on the ecology, distribution, and evolution of these

lineages. Epiloci were pinpointed that correlated with environmental variables (water availability, temperature) suggesting that stable epigenetic divergence led to persistent ecological differences, and set the stage for species-specific genetic patterns to accumulate in response to further selection and/or drift.

Recent studies in our lab have show that epigenetic mechanisms also have a natural role to play in animal transitions into environmental stress-induced states of hypometabolism and this suggests to us the possibility that epigenetic controls will be identified more and more often as significant regulators of animal responses to both short and prolonged environmental change. Our initial work focused on chromatin modification as a means of global inhibition of transcription during winter hibernation in ground squirrels (Morin and Storey, 2006). Histone proteins alter chromatin structure and gate access to DNA by the transcriptional machinery. Increased methylation of lysine residues on histones leads to a more closed chromatin structure whereas acetylation and phosphorylation open up the DNA-protein structure to allow the transcriptional machinery to bind (Jenuwein and Allis, 2001). Analysis of ground squirrel skeletal muscle during torpor showed significant histone modifications that would repress transcription during torpor by creating a more closed chromatin structure. Specifically, acetylated histone H3 (Lys 23) and phosphorylated histone H3 (Ser 10) contents were reduced by 25% and 40%, respectively, during torpor and this correlated with increased protein levels and activity of histone deacetylase (HDAC) (Morin and Storey, 2006). The same mechanism of transcriptional silencing was identified during anoxia-induced metabolic rate depression in turtles (*T. s. elegans*). Transcript and protein levels of five HDACs increased by 1.3-4.6 and 1.7-3.5 fold, respectively, in turtle skeletal muscle during 20 h anoxic submergence. Total HDAC activity also rose by 1.5-fold and levels of acetylated histone H3 decreased by 40-60% (Krivoruchko and Storey, 2010b). Epigenetic regulation also contributes to chromatin remodeling for long term gene silencing during aestivation of green-striped burrowing frogs, *Cyclorana alboguttata* (Hudson et al. 2008). A comparison of mRNA abundance in cruralis muscle of control versus 6-month aestivated frogs found significantly increased transcript levels of transcriptional co-repressor SIN3A and DNA cytosine-5-methyltransferase 1 (by 1.7- and 3.5-fold, respectively), two genes whose protein products have established roles in gene silencing.

6.2 MicroRNA control of mRNA translation

Our understanding of the control of gene expression in cells has changed greatly in recent years with the discovery of the regulatory roles played by various classes of small RNAs. In particular, microRNAs are proving to have major involvement in regulating mRNA transcripts outside the nucleus. MiRNAs are small non-coding transcripts (19-25 nucleotides long) that bind to target mRNAs to regulate translation in normal, stressed and disease states (Filipowicz et al., 2008; Bartel, 2009). A perfect sequence match between the miRNA and its target tends to direct mRNA into degradation pathways, whereas an imperfect match results in translational inhibition via storage in cytoplasmic P-bodies. In recent studies with both hibernating mammals and freeze tolerant frogs, we have found that levels of key miRNA species change significantly when animals enter hypometabolism, suggesting that they have key roles to play in sequestering and preserving mRNA transcripts during stress-induced hypometabolism. In 13-lined ground squirrels, several microRNAs were differentially expressed in kidney, skeletal muscle and heart of when animals entered torpor (Morin et al., 2008). Furthermore, the amount of Dicer, one of two main enzymes involved in microRNA processing increased during torpor. Significant changes in miR-16 and miR-21 levels in liver and skeletal muscle also occurred in response to freezing in wood frogs (Figure 3) (Biggar et al., 2009). Taken together, the two studies suggest that miRNA regulation of mRNA transcription will prove to be a principle of hypometabolism with two consequences: (a) contributing to global translational suppression by sequestering transcripts away from ribosomes, and (b) preserving and

storing transcripts over prolonged periods of hypometabolism so that mRNA is immediately available again for translation when animals arise from dormant states. Furthermore, actions of specific miRNA types may be key to the inhibitory control of selected cell functions during hypometabolism. For example, the cell cycle is highly energy expensive and a target for strong suppression when organisms enter hypometabolism (Biggar and Storey, 2009). Several miRNA species are known to regulate cell cycle genes; e.g. miR-15a and miR-16 target the mRNA transcripts of proteins associated with the first gap phase including cyclin D1, cyclin E, cdc25a, checkpoint kinase 1 and E2F (Kaddar et al., 2009). Indeed, the power of miRNA regulation was shown in studies where cells were transfected to express high levels of miR-16; this resulted in elevated numbers of quiescent cells and reduced numbers of cells in S, G2 and M phases (Linsley et al., 2007). The results for wood frogs (elevated levels of miR-16 in liver, a proliferating tissue) suggest a natural role for miR-16 in cell cycle arrest in freeze-induced hypometabolism and, together with the results from mammalian hibernators, indicate that miRNAs will prove to be a whole new level of regulatory control in animal response to environmental stress. Indeed, changes in miRNA patterns may potentially be developed into biomarkers of cells under stress that could be used to indicate individuals and populations that are under pressure from altered environments such as due to climate change.

[place Figure 3 about here]

Figure 3. (A) Synthesis of microRNA. Primary transcripts are transcribed by RNA polymerase II and processed by ribonucleases (Drosha, Dicer) into single-stranded mature microRNAs. These then join microRNA-induced silencing complex (miRISC) and bind to mRNA transcripts at their 3'-UTR to repress translation. (B) Relative levels of miR-16, miR-21 and Dicer protein in liver and skeletal muscle of frozen wood frogs (24 h at -3°C) compared with 5°C acclimated controls (set to 1). Compiled from Biggar and Storey (2009) and Biggar et al. (2009).

6.3 Tracing signal transduction pathways

The components and typical targets of multiple signal transduction pathways that link sensing of environmental change (generally by cell surface receptors) to downstream actions (e.g. changes in enzyme function, ribosomal translation, gene expression, etc.) are now well known, particularly in mammalian systems. These signaling pathways are highly conserved across the animal kingdom and detection of their activation can be another good diagnostic of an animal system under stress. Indeed, several examples of the activation of mitogen-activated protein kinase (MAPK) signaling cascades in response to anoxia, freezing, or osmotic stresses in amphibians and reptiles are now known (Cowan and Storey, 2003). Therefore, one way to initiate a search for the effects of environmental change on organisms is to evaluate one or more key components of signaling cascades, and when positive responses to stress are found, a follow-up with analyses of both upstream and downstream members of the cascade as well as assessment of known target proteins/enzymes can lead to identification of the major metabolic actions that are triggered by the environmental signal. In a recent example of this approach, we analyzed the responses of the extracellular signal-regulated kinase (ERK) signaling cascade (one member of the MAPK superfamily) to whole body dehydration by African clawed frogs, *Xenopus laevis*. In their native environment, these frogs can experience seasonally arid conditions that require metabolic responses to ameliorate the effects of dehydration. Figure 4 shows the coordinated response by multiple pathway components that occurred in lung when frogs were challenged with medium and high levels of dehydration (Malik and Storey, 2009). All three tiers in the MAPK cascade responded

positively to dehydration with the amounts of phosphorylated active kinases increasing for the initiating MAPK kinase kinases (c-Raf, MEKK), the MAPK kinase (MEK1/2), and finally the MAPK (ERK1/2). Two downstream targets of ERK2 also showed robust increases in amount of active phosphorylated protein: the p90 ribosomal S6 kinase (RSK^{Ser380}) and the transcription factor STAT3^{Ser727} (not shown). RSK activation also led to strong phosphorylation of its target, the S6 ribosomal protein. Furthermore, we found a strong conserved activation of the ERK cascade in most *X. laevis* tissues in response to dehydration (Malik and Storey, 2009). The ERK cascade was also powerfully activated by stress stimuli in heart of *Rana ridibunda* (Gaitanaki et al., 2003). In *X. laevis*, activation of ERK signaling during dehydration was particularly prominent in lung, an organ that is highly susceptible to respiratory water loss. Although not yet experimentally tested, it is interesting to note that the expression of mucin genes in the mammalian respiratory tract is under ERK1/2 control (Choi et al., 2009). This might suggest that ERK activation in lung of dehydrated *X. laevis* might mediate changes in the amount or composition of mucins in airway epithelia to contribute to limiting respiratory water loss.

[place Figure 4 about here]

Figure 4. Response of the ERK signaling pathway to whole body dehydration in lung of African clawed frogs, *Xenopus laevis*. (A) Schematic showing the ERK pathway members analyzed and their positions in the 3-tier signaling cascade that typifies the MAPK family. (B) Relative changes in protein or phosphoprotein (p-) levels, compared with controls (set to 1), in response to medium or high dehydration (16.6 ± 1.59 % or 28.0 ± 1.6 % of total body water lost, respectively) of frogs. Immunoblotting was used to analyze MAPK family members including total protein levels of MEKK, MEK1/2 and ERK2 and phosphorylated active forms: p-cRaf^{Ser338}, p-MEK1/2^{Ser217/221} and p-ERK^{Thr202/Tyr204}. Downstream target proteins phosphorylated by ERK2 included p-p90 ribosomal S6 kinase (RSK)^{Ser380}, total S6 ribosomal protein and p-S6^{Ser235/236}. Data are means ± SEM, n=3-6 independent trials. Compiled from Malik and Storey (2009).

6.4 Transcription factors and identification of stress-responsive genes

Transcription factors bind to DNA at specific sites in the promoter region of genes and activate gene transcription. Typically each individual transcription factor regulates the expression of a select group of genes whose protein products are involved in a specific cell function. Hence, identification of the particular transcription factors that respond to a stress provide another good way of determining which cellular functions are important in the adaptive response to stress. The recent development of array screening technologies that can measure levels of activated transcription factors in cell nuclei has provided a powerful way to screen for those factors that are more active under stress conditions and, thereby, identify “cassettes” of genes that are putatively up-regulated in a coordinated way in response to the imposed stress (Storey, 2008). Figure 5 shows the application of this method to analyzing gene expression responses by turtle liver to 5 h anoxia exposure (Krivoruchko and Storey, 2010c). We chose to evaluate the possible role of the nuclear factor κB (NF-κB) transcription factor in anoxia responsive gene expression due to its known link with antioxidant defense. NF-κB is a heterodimer (p50 and p65 subunits) that is maintained in an inactive state in the cytoplasm by binding to an inhibitory protein, IκB. Dissociation of IκB (after it is phosphorylated by an IκB kinase) allows the NF-κB dimer to migrate to the nucleus where binds to specific sites in the promoter region of genes and activates their transcription. The data in Figure 5 show five different ways in which an activation of NF-κB transcriptional activity can be

evaluated: (a) the amount of phosphorylated I κ B (rose 2-fold in anoxia), (b) mRNA transcript levels of p50 and p65 (increased 2.2-2.9 fold), (c) levels of p50 and p65 protein (increased 1.4-2.3 fold), (d) amount of p50 and p65 protein in the nucleus (increased 1.7-7.3 fold), and (e) p50/p65 binding to DNA (increased 1.9 fold). Furthermore, all five of these agree with the conclusion that NF- κ B is activated by anoxia in turtle liver. In practise, changes in the amount of phosphorylated I κ B or in NF- κ B binding to DNA are often the easiest to measure experimentally. This clear evidence of NF- κ B activation then provides justification for analyzing the anoxia-responsiveness of various genes under NF- κ B control to identify the key targets that help to provide protection from stress. Of particular interest, NF- κ B controls selected antioxidant proteins and the three that we tested were significantly elevated under anoxia: ferritin (an iron-binding protein) and both the Cu/Zn (cytoplasmic) and Mn (mitochondrial) forms of superoxide dismutase. This again highlights the importance of antioxidant defenses for long term viability under stress conditions. Macromolecules need to be protected under anoxia because the capacity to replace damaged proteins using ATP-expensive biosynthesis is strongly suppressed in the hypometabolic state. In addition, an expanded search of NF- κ B targets known from other systems identified elevated levels of two anti-apoptotic proteins (Bcl-2 and Bcl-xL) in liver of anoxic turtles. This is an important finding as it represents some of the very first evidence that inhibition of apoptosis is important for natural anoxia tolerance. Damage to cells by oxygen lack frequently triggers cell death but this would clearly be detrimental for species that can endure cycles of anoxia naturally. The increase in anti-apoptotic markers indicates that animals that are naturally stress tolerant can block apoptosis while they adjust or adapt to the stress condition.

[place Figure 5 about here]

Figure 5. Activation of the NF- κ B pathway signal transduction pathway in response to 5 h of anoxia exposure in liver of turtles, *T. s. elegans*. Under normal conditions, the NF- κ B heterodimer (p50 and p65 proteins) is retained in the cytoplasm bound with its inhibitor protein, I κ B. In response to a stimulus (e.g. anoxia), I κ B kinase is activated and phosphorylates I κ B which then dissociates and is ubiquitinated and degraded. NF- κ B is then free to move to the nucleus and activate transcription of various target genes. Compiled from Krivoruchko and Storey (2010c,d).

7. Concluding remarks

Since the time that ectothermic vertebrates first appeared on land, they have been required to adapt to changes in a wide range of environmental parameters, including many episodes of climate change during which some species have prospered whereas others have faced extirpation or extinction. Our present day concern is with the rapid pace of climate change and with the fact that human activities are a major causative factor in the current episode of climate change, affecting not just ourselves but all organisms on earth. Ectothermic vertebrates will be affected by climate change in ways that include direct effects of heat/cold and changing patterns of water availability on survival and reproductive capacity as well as secondary effects on the availability of food and shelter and competition with other species. All organisms have a variety of biochemical strategies that they can draw on to accomplish adaptation to new environmental realities although some species (such as those that are used to highly stable or highly specialized environments) are inherently poorer at this than others. Cold-hardy reptiles and amphibians living at high latitudes already deal with wide variation in seasonal parameters and, overall, may be better able to implement biochemical

adaptations to deal with a changing climate than comparable tropical species. The molecular mechanisms available for adaptation to environmental stress are many; here we have discussed examples such as proliferation of protectants (low molecular weight metabolites, chaperone proteins), enhanced antioxidant defenses, quantitative changes in gene and protein expression, differential regulation of enzymes, transcription factors, and signaling pathways, epigenetic and post-transcriptional control of genes, and the synthesis of novel proteins. A number of these also have the potential to be effective markers of organisms under stress and we have outlined some of the procedures that can be utilized to search for and identify stress-responsive biochemical adaptations. The potential for human intervention to directly enhance/alter the adaptive strategies of reptiles and amphibians in nature is low but a thorough understanding of how animals adapt to stress gives us the tools to understand what the key stressors are (and perhaps how we can ameliorate them), what the critical adaptations are, how to search for and evaluate molecular adaptations, and what biochemical parameters are most effective markers of stress.

Acknowledgements

We thank the many members of our lab who have contributed to unraveling the mysteries of amphibian and reptile biochemical adaptation to environmental stress including those whose work is featured here: C.A. Dieni, A.I. Malik, A. Krivoruchko, and K.K. Biggar. Research in the Storey lab is supported by NSERC Canada and a Canada Research Chair to KBS.

References

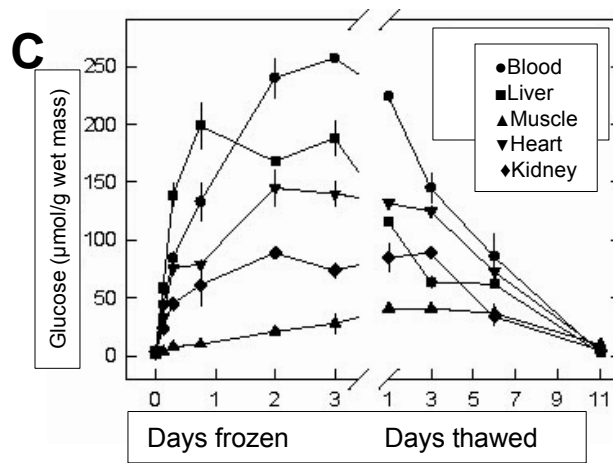
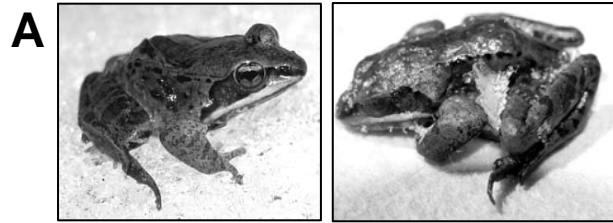
- Angers, B., Castonguay, E. and Massicotte R. (2010) Environmentally induced phenotypes and DNA methylation: how to deal with unpredictable conditions until the next generation and after. *Molecular Ecology* 19, 1283-1295.
- Aubret, F. and Shine, R. (2010) Thermal plasticity in young snakes: how will climate change affect the thermoregulatory tactics of ectotherms? *Journal of Experimental Biology* 213, 242-248.
- Baker, P.J., Costanzo, J.P., Iverson, J.B. and Lee, R.E. (2003) Adaptations to terrestrial overwintering of hatchling northern map turtles, *Graptemys geographica*. *Journal of Comparative Physiology B* 173, 643-651.
- Balinsky, J.B. (1981) Adaptation of nitrogen metabolism to hyperosmotic environment in *Amphibia*. *Journal of Experimental Zoology* 215, 335-350.
- Bartel, D. (2009) MicroRNAs: target recognition and regulatory functions. *Cell* 136, 215-233.
- Bell, R.A.V. and Storey, K.B. (2010) Phosphorylation of liver glutamate dehydrogenase: role in mammalian hibernation. *Comparative Biochemistry and Physiology B* 157, 310-316.
- Berman, D.I., Leirikh, A.N. and Mikhailova, E.I. (1984) Winter hibernation of the Siberian salamander *Hynobius keyserlingi*. *Journal of Evolutionary Biochemistry and Physiology* 1984(3), 323-327. (in Russian with English summary)
- Bickler, P.E. and Buck, L.T. (2007) Hypoxia tolerance in reptiles, amphibians, and fishes: life with variable oxygen availability. *Annual Review of Physiology* 69, 145-170.
- Biggar, K.K. and Storey, K.B. (2009) Perspectives in cell cycle regulation: Lessons from an anoxic vertebrate. *Current Genomics* 10, 573-584.
- Biggar, K., Dubuc, A. and Storey, K.B. (2009) MicroRNA regulation below zero: Differential expression of miRNA-21 and miRNA-16 during freezing in wood frogs. *Cryobiology* 59, 317-321.
- Bosch, J., Carrascal, L.M., Duran, L., Walker, S. and Fisher, M.C. (2007) Climate change and outbreaks of amphibian chytridiomycosis in a montane area of Central Spain; is there a link? *Proceedings of the Royal Society B: Biological Sciences* 274, 253-260.

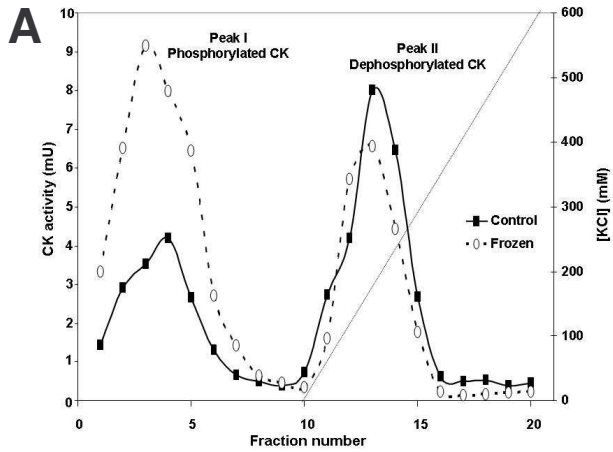
- Choi, H.J., Chung, Y.S., Kim, H.J., Moon, U.Y., Choi, Y.H., Van Seuningen, I., Baek, S.J., Yoon, H.G. and Yoon, J.H. (2009) Signal pathway of 17beta-estradiol-induced MUC5B expression in human airway epithelial cells. *American Journal of Respiratory Cell and Molecular Biology* 40, 168-178.
- Costanzo, J.P. and Lee, R.E. (2005) Cryoprotection by urea in a terrestrially hibernating frog. *Journal of Experimental Biology* 208, 4079-4089.
- Costanzo, J.P., Lee, R.E. and Ultsch, G.R. (2008) Physiological ecology of overwintering in hatchling turtles. *Journal of Experimental Zoology A* 309, 297-379.
- Cowan, K.J. and Storey, K. B. (2003) Mitogen-activated protein kinases: new signaling pathways functioning in cellular responses to environmental stress. *Journal of Experimental Biology* 206, 1107-1115.
- Dieni, C.A. and Storey, K.B. (2009) Creatine kinase regulation by reversible phosphorylation in frog muscle. *Comparative Biochemistry and Physiology B* 152, 405-412.
- Dynesius, M. and Jansson, R. (2000) Evolutionary consequences of changes in species' geographical distributions driven by Milankovitch climate oscillations. *Proceedings of the National Academy of Science USA* 97(16), 9115-9120.
- Eddy, S.F. and Storey, K.B. (2008) Comparative molecular physiological genomics: heterologous probing of cDNA arrays. *Methods in Molecular Biology* 410, 81-110.
- Filipowicz, W., Bhattacharyya, S., Sonenberg, N. (2008) Mechanisms of post-translational regulation by microRNAs: are the answers in sight? *Nature Reviews Genetics* 9, 102-114.
- Fletcher, G. L., Hew, C. L. and Davies, P. L. (2001) Antifreeze proteins of teleost fish. *Annual Review of Physiology* 63, 359-390.
- Gaitanaki, C. Konstantina, S., Chrysa, S. and Beis, I. (2003) Oxidative stress stimulates multiple MAPK signalling pathways and phosphorylation of the small HSP27 in the perfused amphibian heart. *Journal of Experimental Biology* 206, 2759-2769.
- Gibbons, J.W. and Nelson, D.H. (1978) The evolutionary significance of delayed emergence from the nest by hatchling turtles. *Evolution* 32, 297-303.
- Gregory, P.T. and Stewart K.W. (1975) Long-distance dispersal and feeding strategy of the red-sided garter snake (*Thamnophis sirtalis parietalis*) in the Interlake of Manitoba. *Canadian Journal of Zoology* 53, 238-245.
- Hayes, T.B., Falso, P., Gallipeau, S. and Stice, M. (2010) The cause of global amphibian declines: a developmental endocrinologist's perspective. *Journal of Experimental Biology* 213, 921-933.
- Herbert, C.V. and Jackson, D.C. (1985) Temperature effects on the response to prolonged submergence in the turtle *Chrysemys picta bellii*. II. Metabolic rate, blood acid-base and ionic changes, and cardiovascular function in aerated and anoxic water. *Physiological Zoology* 58, 670-681.
- Hermes-Lima, M., Storey, J.M. and Storey, K.B. (2001) Antioxidant defenses and animal adaptation to oxygen availability during environmental stress. In: (Storey, K.B. and Storey, J.M., eds.) *Cell and Molecular Responses to Stress*. Elsevier Press, Amsterdam, Vol. 2, pp. 263-287.
- Hewitt, G.M. (1996) Some genetic consequences of ice ages, and their role in divergence and speciation. *Biological Journal of the Linnean Society* 58, 247-276.
- Hillman, S.S., Withers, P.C., Drewes, R.C. and Hillyard, S.D. 2009. *Ecological and Environmental Physiology of Amphibians*. Oxford University Press, Oxford.
- Hochachka, P.W. and Somero, G.N. 1984. *Biochemical Adaptation*. Princeton University Press, Princeton.
- Hudson, N.J., Lonhienne, T.G., Franklin, C.E., Harper, G.S. and Lehnert, S.A. (2008) Epigenetic silencers are enriched in dormant desert frog muscle. *Journal of Comparative Physiology B*

- 178, 729-734.
- Hulin, V., Delmas, V., Girondot, M., Godfrey, M.H. and Guillon, J-M. (2009) Temperature-dependent sex determination and global change: are some species at greater risk? *Oecologia* 160, 493-506.
- Jablonka, E. and Raz, G. (2009) Transgenerational epigenetic inheritance: prevalence, mechanisms, and implications for the study of heredity and evolution. *Quarterly Review of Biology* 84, 131-176.
- Jackson, D.C. and Ultsch, G.R. (2010) Physiology of hibernation under the ice by turtles and frogs. *Journal of Experimental Zoology A* 313, 311-327.
- Jenuwein, T. and Allis, C.D. (2001) Translating the histone code. *Science* 293, 1074-1080.
- Linsley, P., Schelter, J., Burchard, J., Kibukawa, M., Martin, M., Bartz, S., Johnson, J., Cummins, J., Raymond, C., Dai, H., Chau, N., Cleary, N., Jackson, A., Carleton, M. and Lim, L. (2007) Transcripts targeted by the microRNA-16 family cooperatively regulate cell cycle progression. *Molecular and Cellular Biology* 27, 2240-2252.
- Kaddar, T., Rouault, J.P., Chien, W.W., Chebel, A., Gadoux, M., Salles, G., French, M., Magaud, J.P. (2009) Two new miR-16 targets: caprin-1 and HMGA1, proteins implicated in cell proliferation. *Biology of the Cell* 101, 511-24.
- Kearney, M., Shine, R. and Porter, W.P. (2009) The potential for behavioral thermoregulation to buffer 'cold-blooded' animals against climate warming *Proceedings of the National Academy of Science USA* 106, 3835-3840.
- Krivoruchko, A. and Storey, K.B. (2010a) Regulation of the heat shock response under anoxia in the turtle, *Trachemys scripta elegans*. *Journal of Comparative Physiology B* 180, 403-414.
- Krivoruchko, A. and Storey, K.B. (2010b) Epigenetics in anoxia tolerance: a role for histone deacetylases. *Molecular and Cellular Biochemistry* 342, 151-161.
- Krivoruchko, A. and Storey, K.B. (2010c) Molecular mechanisms of turtle anoxia tolerance: A role for NF- κ B. *Gene* 450, 63-69.
- Krivoruchko, A. and Storey, K.B. (2010d) Forever young: mechanisms of anoxia tolerance in turtles and possible links to longevity. *Oxidative Medicine and Cellular Longevity* 3(3), 186-198.
- Kültz, D. (2005) Molecular and evolutionary basis of the cellular stress response. *Annual Review of Physiology* 67, 225-257.
- Lance, V.A. (2009) Is regulation of aromatase expression in reptiles the key to understanding temperature-dependent sex determination? *Journal of Experimental Zoology A* 311, 314-322.
- Lee-Yaw, J.A., Irwin, J.T. and Green, D.M. (2008) Postglacial range expansion from northern refugia by the wood frog, *Rana sylvatica*. *Molecular Ecology* 17(3), 867-884.
- Lutz, P.L. and Milton, S.L. (2004) Negotiating brain anoxia survival in the turtle. *Journal of Experimental Biology* 207(18), 3141-3147.
- Malik, A.I. and Storey, K.B. (2009) Activation of extracellular signal-regulated kinases during dehydration in the African clawed frog, *Xenopus laevis*. *Journal of Experimental Biology* 212, 2595-2603.
- Margesin, R., Neuner, G. and Storey, K.B. (2007). Cold-loving microbes, plants and animals – fundamental and applied aspects. *Naturwissenschaften* 94, 77-99.
- McCaffery, R.M. and Maxell, B.A. (2010) Decreased winter severity increases viability of a montane frog population. *Proceedings of the National Academy of Science USA* 107(19), 8644-8649.
- McCallum, M.L., McCallum, J.L., Trauth, S.E. (2009) Predicted climate change may spark box turtle declines. *Amphibia-Reptilia* 30, 259-264

- McMenamin, S.K. and Hadly, E.A. (2010) Developmental dynamics of *Ambystoma tigrinum* in a changing landscape. *BMC Ecology* 10, 10.
- Mitchell, N.J. and Janzen, F.J. (2010) Temperature-dependent sex determination and contemporary climate change. *Sex and Development* 4(1-2), 129-140.
- Mitchell, N.J., Kearney, M.R., Nelson, N.J. and Porter, W.P. (2008) Predicting the fate of a living fossil: how will global warming affect sex determination and hatching phenology in tuatara? *Proceedings of the Royal Society B: Biological Sciences* 275, 2185–2193.
- Mommsen, T.P. and Storey, K.B. (1992) Hormonal effects on glycogen metabolism in isolated hepatocytes of a freeze-tolerant frog. *General and Comparative Endocrinology* 87, 44-53.
- Morin, P. and Storey, K.B. (2006) Evidence for a reduced transcriptional state during hibernation in ground squirrels. *Cryobiology* 53, 310-318.
- Morin, P., Dubuc, A. and Storey, K.B. (2008) Differential expression of microRNA species in organs of hibernating ground squirrels: a role in translational suppression during torpor. *Biochimica et Biophysica Acta* 1779, 628–633.
- Paun, O., Bateman, R.M., Fay, M.F., Hedren, M., Civeyrel, L. and Chase, M.W. (2010) Stable epigenetic effects impact adaptation in allopolyploid orchids (Dactylorhiza: Orchidaceae). *Molecular Biology and Evolution* 20, in press. doi:10.1093/molbev/msq150
- Pidwirny, M. (2006) *Fundamentals of Physical Geography, 2nd Edition*. <http://www.physicalgeography.net/>.
- Rider, M.H., Hussain, N., Horman, S., Dilworth, S.M. and Storey, K.B. (2006) Stress-induced activation of the AMP-activated protein kinase in the freeze-tolerant frog *Rana sylvatica*. *Cryobiology* 53, 297-309.
- Storey, K.B. (1997). Organic solutes in freezing tolerance. *Comparative Biochemistry and Physiology A* 117, 319-326.
- Storey, K.B. (2002) Life in the slow lane: molecular mechanisms of estivation. *Comparative Biochemistry and Physiology A* 133, 733-754.
- Storey, K.B. (2004a) *Functional Metabolism: Regulation and Adaptation*. Wiley-Liss, Hoboken, NJ.
- Storey, K.B. (2004b) Strategies for exploration of freeze responsive gene expression: advances in vertebrate freeze tolerance. *Cryobiology* 48, 134-145.
- Storey, K.B. (2006) Reptile freeze tolerance: metabolism and gene expression. *Cryobiology* 52, 1-16.
- Storey, K.B. (2007) Anoxia tolerance in turtles: metabolic regulation and gene expression. *Comparative Biochemistry and Physiology A* 147, 263-276.
- Storey, K.B. 2008. Beyond gene chips: transcription factor profiling in freeze tolerance. In: (Lovegrove, B.G., and McKechnie, A.E., eds.) *Hypometabolism in Animals: Hibernation, Torpor and Cryobiology*. University of KwaZulu-Natal, Pietermaritzburg, pp. 101-108.
- Storey, K.B. and Storey, J.M. (1986) Freeze tolerant frogs: cryoprotectants and tissue metabolism during freeze/thaw cycles. *Canadian Journal of Zoology* 64, 49-56.
- Storey, K.B. and Storey, J.M. (1988). Freeze tolerance in animals. *Physiological Reviews* 68, 27-84.
- Storey, K.B. and Storey, J.M. (1990) Facultative metabolic rate depression: molecular regulation and biochemical adaptation in anaerobiosis, hibernation, and estivation. *Quarterly Review of Biology* 65, 145-174.
- Storey, K.B. and Storey, J.M. (1992) Natural freeze tolerance in ectothermic vertebrates. *Annual Review of Physiology* 54, 619-637.
- Storey, K.B. and Storey, J.M. (2004a) Physiology, biochemistry and molecular biology of vertebrate freeze tolerance: the wood frog. In: *Life in the Frozen State* (Benson, E., Fuller, B., and Lane, N., eds.) CRC Press, Boca Raton, pp. 243-274.
- Storey, K.B. and Storey, J.M. (2004b) Metabolic rate depression in animals: transcriptional and translational controls. *Biological Reviews of the Cambridge Philosophical Society* 79, 207-

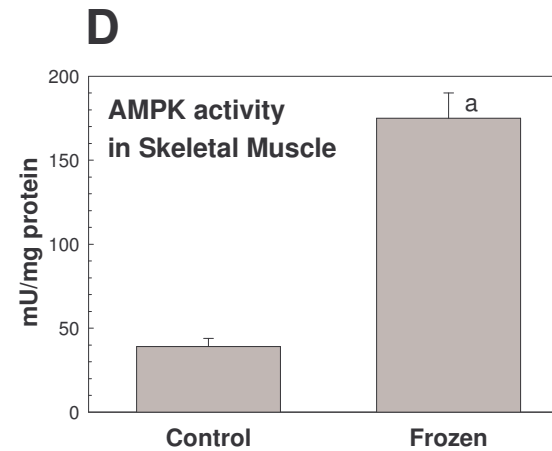
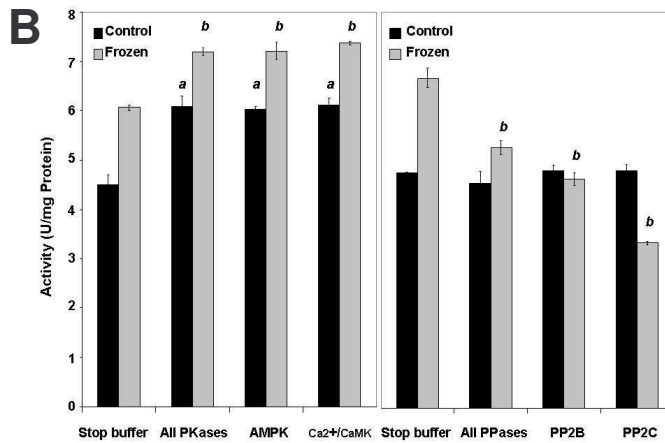
- 233.
- Storey, K.B. and Storey, J.M. (2007) Putting life on 'pause' – molecular regulation of hypometabolism. *Journal of Experimental Biology* 210, 1700-1714.
- Storey, K.B. and Storey, J.M. (2010) Metabolic regulation and gene expression during aestivation. In: (Navas, C.A. and Carvalho, J.E., eds) *Aestivation: Molecular and Physiological Aspects*. Springer, Heidelberg, pp. 25-45.
- Storey, K.B., Storey, J.M., Brooks, S.P.J., Churchill, T.A. and Brooks, R.J. (1988) Hatchling turtles survive freezing during winter hibernation. *Proceedings of the National Academy of Science USA* 85, 8350-8354.
- Stuart, S.N., Chanson, J.S., Cox, N.A., Young, B.E., Rodrigues, A.S., Fischman, D.L. and Waller, R.W. (2004) Status and trends of amphibian declines and extinctions worldwide. *Science* 306(5702), 1783-1786.
- Tattersall, G.J. and Ultsch, G.R. (2008) Physiological ecology of aquatic overwintering in ranid frogs. *Biological Reviews of the Cambridge Philosophical Society* 83(2), 119-140.
- Turner, B.M. (2009) Epigenetic responses to environmental change and their evolutionary implications. *Philosophical Transactions of the Royal Society, London B Biological Sciences* 364(1534), 3403-3418.
- Voituron, Y., Storey, J.M., Grenot, C. and Storey, K.B. (2002) Freezing survival, body ice content and blood composition of the freeze tolerant European common lizard, *Lacerta vivipara*. *Journal of Comparative Physiology B* 172, 71-76.
- Voituron, Y., Heulin, B. and Surget-Groba, Y. (2004) Comparison of the cold hardiness capacities of the oviparous and viviparous forms of *Lacerta vivipara*. *Journal of Experimental Zoology A* 301, 367-373.
- Voituron, Y., Paaschburg, L., Holmstrup, M., Barré, H. and Ramløv, H. (2009) Survival and metabolism of *Rana arvalis* during freezing. *Journal of Comparative Physiology B* 179(2), 223-230.
- Wake, D.B. (2007) Climate change implicated in amphibian and lizard declines. *Proceedings of the National Academy of Science USA* 104, 8201-8202.
- Warren, D.E. and Jackson, D.C. (2007) Effects of temperature on anoxic submergence: skeletal buffering, lactate distribution, and glycogen utilization in the turtle, *Trachemys scripta*. *American Journal of Physiology* 293, R458-R467.
- Yancey, P.H. (2005) Organic osmolytes as compatible, metabolic and counteracting cytoprotectants in high osmolarity and other stresses. *Journal of Experimental Biology* 208, 2819-2830.
- Ziegler, R., Ashida, M., Fallon, A. M., Wimer, L. T., Silver Wyatt, S. and Wyatt, G. R. (1979) Regulation of glycogen phosphorylase in fat body of *Cecropia* silkworm pupae. *Journal of Comparative Physiology* 131, 321-332.
- Zhang, X. (2008) The epigenetic landscape of plants. *Science* 320, 489-492.

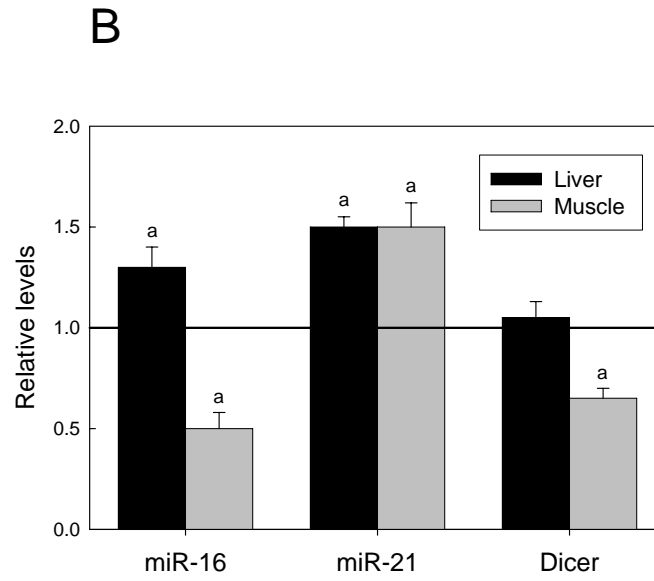
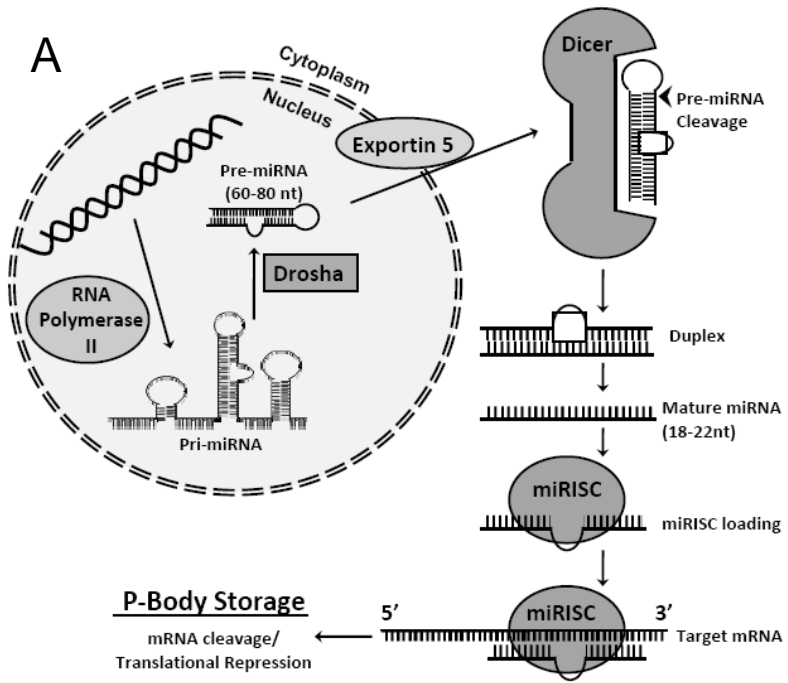


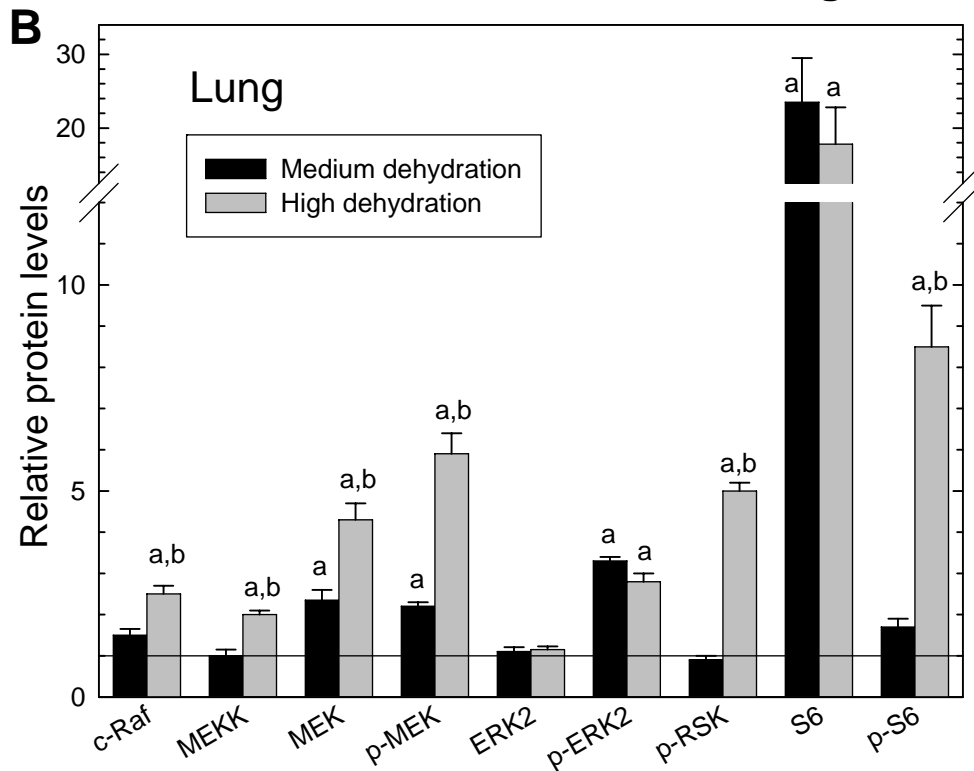
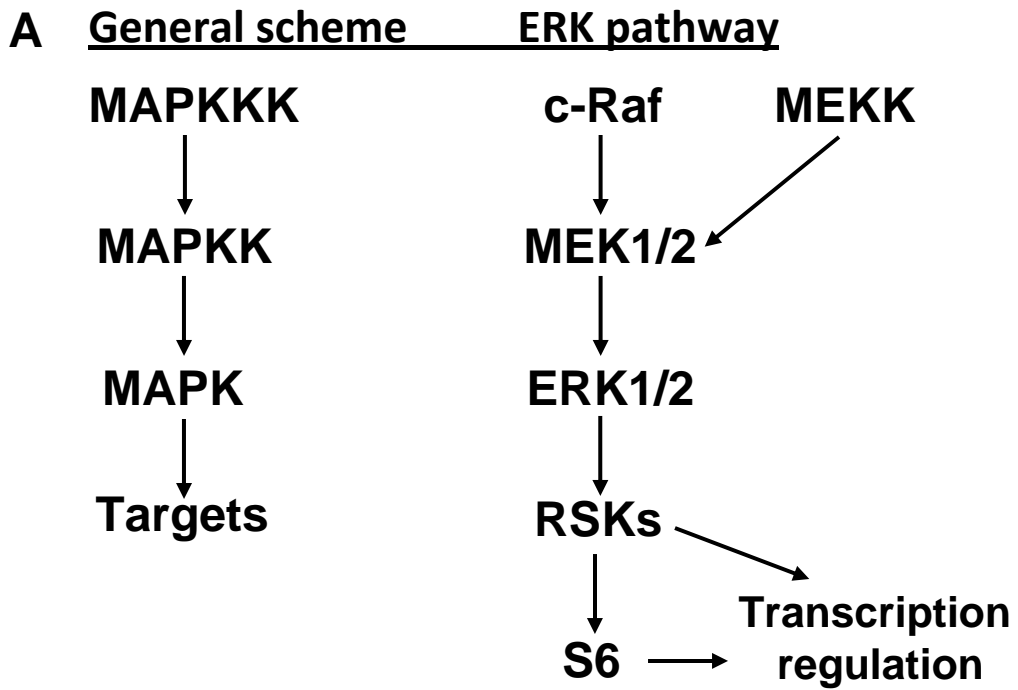


C

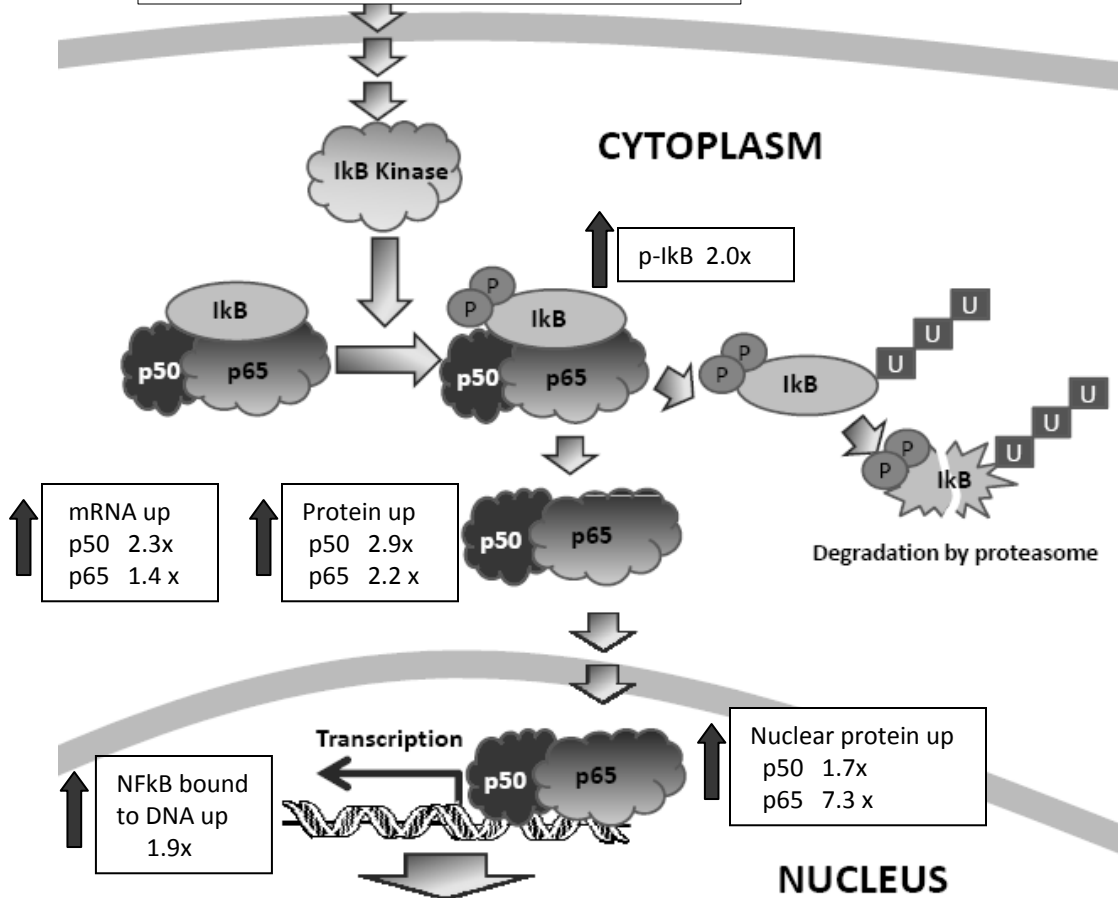
<i>K_m</i> Creatine (mM)		
Incubation	Control	Frozen
Stop	4.52 ± 0.19	3.22 ± 0.09 ^a
Kinase	3.57 ± 0.16 ^a	3.14 ± 0.1
Phosphatase	5.09 ± 0.07 ^a	5.03 ± 0.18 ^b







5 h ANOXIA in TURTLE LIVER



Elevated mRNA levels of downstream genes

Antioxidants		Anti-apoptosis	
Ferritin	1.6x ↑	Bcl-xL	2.8x ↑
Mn SOD	2.5x	Bcl-2	2.4x
Cu/Zn SOD	1.7x		