

Cryotolerance in Lyon

Freezing Lizards

Lizards apply different mechanisms of freeze tolerance to many other animals. Yann Voituron and his group have now revealed the role of mitochondria in avoiding oxidative stress following a re-warming period in winter.



Not just cold feet: Lizard in the snow

How do animals survive the cold of winter? Some migrate to warmer climes but for those that overwinter, the lack of food and low temperatures present special problems. When ambient temperatures fall below zero, animals that can generate their own body heat (endotherms) may choose to brave the cold or to conserve their energy stores by hibernating in a cool, dry spot until spring. But what happens to the ectotherms, animals that possess little or no means of regulating their internal body temperature?

Yann Voituron, Professor of Ecophysiology at the University of Lyon, researches just how solidly frozen such animals can become and the remarkable survival strategies that they have evolved to cope with the physiological stress of freezing body tissues.

Ice within the body

Most temperate ectotherms avoid sub-freezing temperatures by migration or by using insulated hibernation sites, yet some species of terrestrial hibernators, including certain amphibians, reptiles and insects, have developed specific physiological mechanisms that allow them to evade cold injuries during winter. To ensure their survival at sub zero temperatures, these animals have developed two main cold hardiness strategies: freeze avoidance and freeze tolerance. Freeze avoidance entails an extensive supercooling capacity characterised by metabolic adaptations involving the release or masking of potent ice nucleators, the accumulation of low molecular weight carbohydrates and antifreeze proteins that depress freezing point. By con-

trast, the freeze tolerance strategy implies that animals can endure the conversion of a fraction of their body water into ice. This second strategy is characterized by mechanisms such as the production of ice nucleators which allow the initiation of freezing at high sub-zero temperatures and the production of cryoprotectant substances that allow the controlled propagation of ice within the body.

Yann Voituron has been particularly interested in freezing lizards, studying the European common lizard, *Lacerta vivipara*, which exhibits the rare capacity to survive winter by means of both freeze tolerance and freeze avoidance. This highly adaptable species lives in damp habitats such as meadows and heathlands, from sea level to 3,000 metre altitudes, spread out over a huge area from the mountains of northwest Spain to Sakhalin on the Pacific coast, and from northern Spain to beyond the Arctic Circle. During winter, the lizard typically hibernates in shallow sites 2-4 cm beneath the vegetative litter in grass hummocks that have a permanent humidity of around 100%.

The cold hardiness strategy of *L. vivipara* was first studied using radioactive markers to locate overwintering individuals in the field. At the same site, lizards could be in either the supercooled or frozen state and even at ambient temperatures as low as -8°C , unfrozen lizards were found. Yann has subsequently been collecting lizards and freezing them under laboratory conditions at different temperatures, thawing them at different rates and studying their survival capacities. Lizards are capable of surviving the conversion of nearly

50% of their total body water into ice for at least 24 hours, with crystallisation occurring at around -2°C on wet substrata and -4°C when dry. In a supercooled state they can survive for at least 21 days at -3.5°C . Such a rare physiological capacity explains the exceptional survival rates of *L. vivipara* (88–100%, all age classes) even during the extreme cold of winter.

Yann's work is concerned with two principal questions about the freeze tolerance strategies of ectotherms. Firstly, concerning their physiology, what are the underlying mechanisms of this adaptation and what is its metabolic cost? Secondly, in evolutionary terms when, and how often, has this physiological characteristic appeared and what is its adaptive advantage?

The lizards seem to have evolved a particular process that combines several mechanisms. Many freeze-tolerant animals accumulate high concentrations of sugars or polyhydric alcohols that act as cryoprotectants, for example, freeze-tolerant frogs typically use glucose for this function whereas most insects use glycerol or other polyols. However, among reptiles, cryoprotectants are generally lacking and, although the freezing of *L. vivipara* does stimulate a rise in blood glucose levels that increases throughout the winter season, its concentration remains relatively low compared to other freeze tolerant species.

Little sugar and no alcohol

Cold-hardy species, such as cold-water fish and freeze-avoiding insects employ special antifreeze proteins to manage ice, where their primary role is to adhere to the growth planes of microscopic ice crystals

and prevent their growth to a size that could cause injury. However, *L. vivipara* does not seem to use such antifreeze proteins.

Another aspect of the lizard's ice-cold physiology that Yann has investigated is its capacity to minimise tissue damage from its exposure to sub zero temperatures, particularly associated with oxidative stress. Whether in a supercooled or frozen state, lizards continue to obtain energy through the oxidation of metabolic compounds. Indeed, there is evidence of an activation of aerobic metabolic pathways between 0.5°C and -1.5°C. However, there is limited oxygen availability to tissues, compelling the lizards to cope with potential oxidative stress during the transition from ischemic/anoxic conditions to reperfusion with aerated blood during recovery when temperatures increase again. In particular, there is the risk that the generation of reactive oxygen species (ROS) can initiate free radical chain reactions damaging DNA, proteins and lipids.

Yann determined whether antioxidant defences, including low molecular weight free radical scavengers and enzymatic antioxidant systems, are implicated in the lizard's survival when facing sub zero temperatures. He monitored the activities in muscle and the liver of the main antioxidant enzymes, superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT), during either supercooling or freezing induced by exposure to -2.5 °C for 20 hours and 24 hours after thawing at 5 °C.

Focus on mitochondria

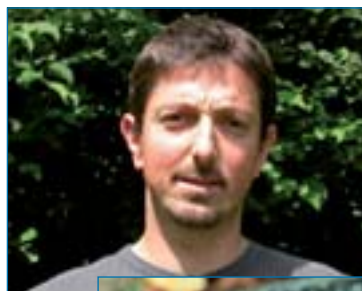
Supercooling induced a significant increase in the total SOD and GPx activity in muscle, but freezing had almost no effect on enzyme activity, either in muscle or in liver. By contrast, thawed lizards exhibited higher GPx activity in both organs and a significant decrease in liver catalase activity. Hence, supercooling, but not freezing, triggers activation of the antioxidant system. This is probably in anticipation of the overgeneration of oxyradicals when the temperature increases while thawing or at the end of supercooling. To assess the disturbance of the balance of pro-oxidant/anti-oxidant mechanisms, the oxidative damage to DNA in cells was assessed. This suggested that the lizard's antioxidant system is fairly efficient since no oxidative damage to DNA could be detected whatever the physiological state of the lizards. Nevertheless,

the changes in the lizard's antioxidant system were still relatively small compared to other species so Yann postulated the existence of other cellular adaptive mechanisms to avoid oxidative stress.

Mitochondria are a significant source of ROS and there are mechanisms to limit ROS generation within mitochondria, including alterations of the mitochondrial membrane, increases in mitochondrial volume density, and synthesis of specific isoenzymes and stress proteins.

In his latest paper (*J. Exp. Biol.* 211: 1456-62), Yann details his discovery of a novel mitochondrial protein that could reduce ROS production in reptilian mitochondria under sub zero temperatures. Mitochondrial functioning differs between endotherms and ectotherms: endotherms have a 5-10 times higher mitochondrial oxygen consumption rate while mitochondria from ectotherms are less proton leaky. To Yann, this suggested that there were differences in the fatty acid composition of mitochondrial membrane phospholipids and/or different degrees of modulation of mitochondrial proton leakage by proteins such as uncoupling proteins (UCPs) that are found in the

inner mitochondrial membrane. UCPs can modulate the mitochondrial proton motive force, a key factor influencing ROS production in the mitochondrial



Yann Voituron (above) and *Lacerta vivipara*

respiratory chain. Furthermore, exogenous superoxide has been reported to activate UCP-mediated uncoupling *in vitro*. Therefore, the uncoupling activity of UCPs *in vivo* may play a crucial role in protecting tissues from oxidative stress during periods when over generation of ROS is expected.

Interestingly, UCPs are also implicated in the hibernation of endotherms. UCP1 converts energy directly into heat in hibernating mammals by providing an alternative pathway for protons to re-enter the mi-

tochondrial matrix. The 'proton circuit' that links the respiratory chain to ATP synthesis is short-circuited by UCP1, allowing respiration to proceed in the absence of ATP synthesis. Free fatty acids activate mammalian UCP1 in brown fat, overriding inhibition by GDP/ADP.

Radical restriction

Yann and his colleagues used RT-PCR to look for the potential existence of an uncoupling protein (UCP) in *Lacerta vivipara* and successfully discovered the first reptilian UCP homologue. Expression of the reptilian uncoupling protein (repUCP) gene was ubiquitously detected in 4°C cold-acclimated lizard tissues and upregulated in muscle tissues following a 20 hour exposure to sub zero temperatures in a supercooling state or after thawing. Similar patterns of expression were observed for repUCP co-activators, suggesting that mechanisms regulating UCP expression may be conserved between mammals (endotherms) and reptiles (ectotherms).

The functionality of repUCP was assessed by measurements of respiration and hydrogen peroxide production from muscle mitochondria. Proton leakage through the inner mitochondrial membrane, which partially uncouples phosphorylation from oxidation, can be regulated through inducible UCP-dependent processes. UCP activity can be inhibited by purine nucleoside diphosphates. A guanosine diphosphate-sensitive non-phosphorylating respiration resulted in an increase in endogenous mitochondrial hydrogen peroxide production, indicating that there was an inhibition of extra proton leakage mediated by a UCP. Hence in situations of stressful oxidative reperfusion following thawing, there does appear to be a physiological role for repUCP in superoxide limitation by lizard mitochondria.

In addition to its antioxidant role, Yann suggests that repUCP is an inducer of fatty acid oxidation. Overwintering lizards have increased levels of free fatty acids in addition to increases in blood glucose. In his ongoing search for a 'global' cold hardiness strategy in *L. vivipara*, Yann currently thinks that at sub zero temperatures the lizard preferentially uses fatty acids as a metabolic substrate, preserving the blood glucose for its cryoprotective functions.

In the longer term, Yann believes that understanding how *L. vivipara* survives the cold may help in the development of techniques that avoid the cryopreservation injury associated with organ transplantation.

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